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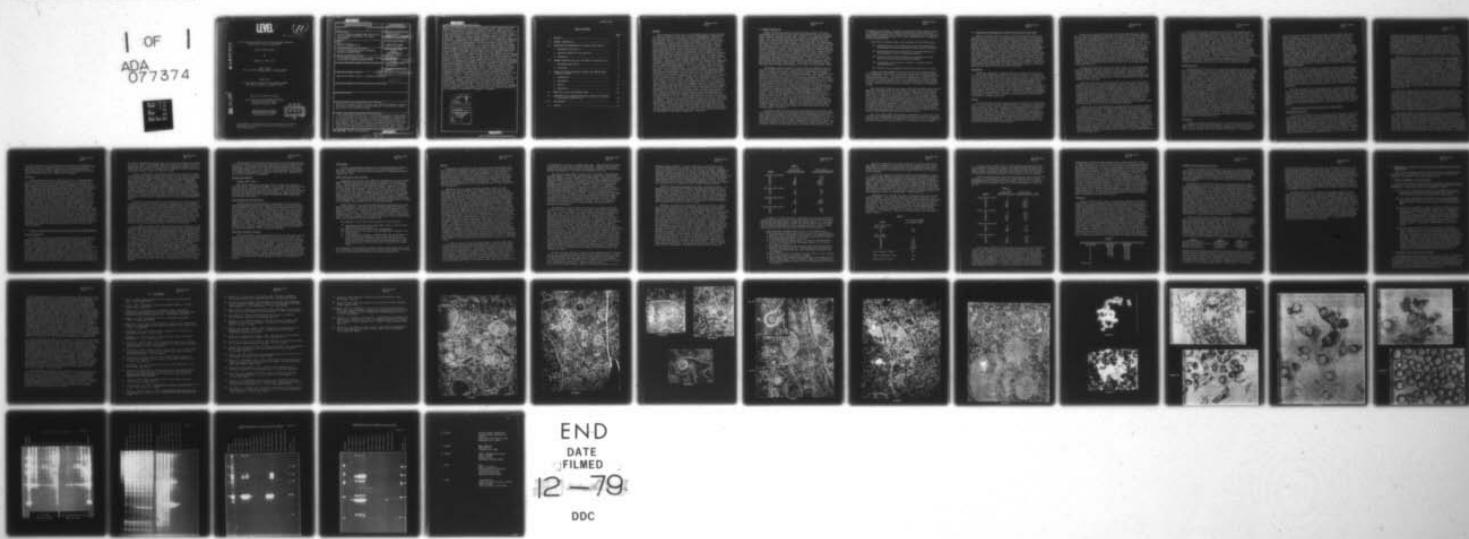
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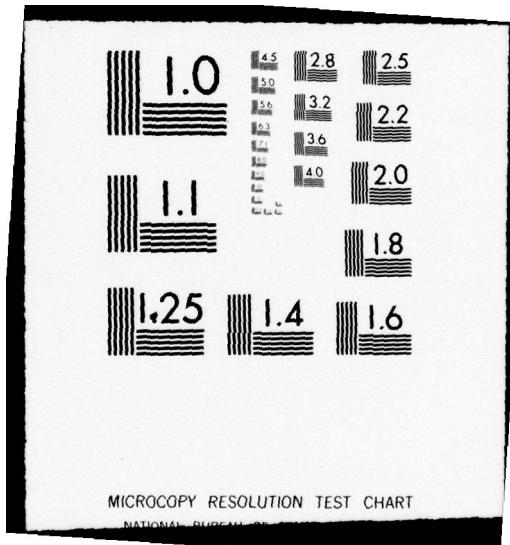
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In vitro Studies of Sandfly Fever Viruses and Their Potential
Significance for Vaccine Development

Annual Progress Report

by

Jonathan F. Smith, Ph.D.

January 1979
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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The replication of Punta Toro virus (Phlebotomus group) has been studied in cultures of BHK-15 or vero cells in terms of morphogenesis, immunofluores-cent localization of viral antigens, and by an electrophoretic analysis of virus-specific proteins. The synthesis of Karimibad virus proteins has also been examined. By thin-section electron microscopy procedures, it has been established that the maturation of Punta Toro virus occurs exclusively on smooth internal membranes and predominantly in perinuclear areas. Budding is initiated as early as 15 hours after infection and presumably earlier.

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Particulate nucleocapsids are not observed prior to their condensation into budding particles at the surface of cytoplasmic vesicles (Golgi vesicles). Viral ribonucleoprotein complexes apparently bind only to membranes which have been modified by viral envelope proteins which are visible as spikes on the lumen (contralateral) side of vesicular membranes; areas of vesicular membrane immediately adjacent to budding complexes manifest neither of these viral structures. These observations suggest some form of transmembranyl recognition, presumably transmembranyl viral glycoprotein(s). Virus release from infected cells is apparently accomplished by exocytosis (fusion of virus-containing vesicles with the plasma membrane). It is not yet known whether this process also results in the integration of virus-specific proteins into cell surface membranes. Virus-specific antigens were found by indirect immunofluorescence to be distributed throughout the cytoplasm, but with a pronounced perinuclear concentration. Fluorescent inclusion bodies were also observed within the nuclei of infected cells, suggesting some role, as yet unknown, for the nucleus in the replication of this virus. Although virus replication gradually inhibits host cell-specific protein synthesis, this inhibition is both delayed and incomplete, and this situation has impeded the study of viral protein synthesis. However, the utilization of immunoprecipitation or immune-adsorption techniques has largely overcome these problems. Data are presented in which these procedures have been used to identify and characterize the virus-specific polypeptides of Punta Toro and Karimibad viruses. Polypeptide profiles of Punta Toro virus structural proteins appear similar to the virus-specific proteins isolated from whole-cell lysates; however, several polypeptides have been immunoprecipitated from Karimibad virus-infected cells which have not yet been detected in virions. These polypeptides may constitute non-structural or precursor proteins, or structural proteins present in virions in relatively low amounts. The significance of these data is discussed, both here and in the accompanying proposal, with respect to the theoretical coding capability of these viruses and the mechanisms which could control the expression of their viral genomes. Preliminary data is also included on the growth of cells and viruses in microcarrier cultures. The potential of this mass culture technique for the production of large amounts of virus and virion antigens required for the development of experimental immunogens is discussed.

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1. ABSTRACT

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II. Underline: General Introduction

Despite the fact that among the large and diverse arbovirus class the Bunyaviridae constitute by far the largest group and that many are significant human pathogens, knowledge of their basic virology is at a relatively elemental stage. This lack of basic knowledge also extends to the phlebotomus group which is the largest group among the Bunyaviridae and of which at least five members are known to cause human disease. On the other hand, the epidemiology and vector relationships of the phlebotomus agents have been fairly well studied, most notably by Tesh. Similarly, the clinical syndrome of the sandfly fever is also well described both from naturally occurring cases and from experimentally induced infections in humans. Sabin demonstrated as early as 1944 that both the Sicilian and Naples strain could be attenuated by mouse brain passage and that such brain preparations could be successfully used as vaccines insofar as vaccinated individuals did not develop clinical disease and were resistant to experimental challenge with the homologous agent. Sabin's studies show quite conclusively that protection from these diseases via vaccination is a realistic goal although these mouse brain preparations are no longer acceptable due to potential neuroparalytic complications. These aspects of sandfly fever have been extensively reviewed and documented in the first research proposal (1978).

The knowledge which is presently lacking, which will complement the data referred to above, and which will be necessary to understand these viruses as agents of disease and to design, produce, and test experimental immunogens relates to the basic virology of the phlebotomus agents. Data are needed on the intracellular replication of these viruses, on the logistical problems of preparing and purifying virus grown in cell substrates acceptable for vaccine production, on the controls imposed upon virus gene expression and on the synthesis of virus-coded proteins and antigens - both in terms of their function in virion maturation and as antigens which potentially could offer protection. For example, whether or not virion antigens exist at the surface of infected cells is important not only in terms of disease pathogenesis, i.e. whether infected cells could be recognized in vivo by immune surveillance mechanisms, but also to determine if cytotoxicity assays are viable candidates to monitor T cell activity. Also, purified glycoproteins could presumably be functional as a subunit vaccine, but the manner in which they are presented as an antigen is expected to be critical. Morein (1) has recently shown that as little as one half of one microgram of the purified virion glycoproteins of Semliki Forest Virus, given as a single injection, will protect mice injected with an otherwise lethal dose of virus. However, this vaccination was only successful if the proteins are given in the form of protein micelles or protein-lipid micelles - completely solubilized protein was without protective effect (see renewal proposal, section III.

The central points which are presently being addressed in our studies are:-
1) to determine the optimal procedures for production and purification of phlebotomus viruses and antigens, 2) to characterize the morphogenesis patterns of these agents and the intracellular localization of virus antigens, 3) to identify virus-specific proteins and to examine their synthesis and function both as structural proteins of the virus and as components of infected cells, and 4) to determine the manner in which expression of the viral genome is controlled. The last point refers to the fact that we have identified more proteins than there

are segments in the genome. Therefore, either polycistronic messengers result in the production of precursor polyproteins which are cleaved during post-translational processing (as in enteroviruses or alphaviruses),or multiple but monocistronic messengers originate from single RNA segments (as in rhabdoviruses and paramyxoviruses).

In the past five months progress has been made in the following areas:

(1) Identification of virion structural proteins and glycoproteins and characterization by electrophoresis and isoelectric focusing

(2) Characterization of the intracellular virus-coded proteins and the termination of host cell-directed synthesis

(3) Characterization of the morphology and morphogenesis of Punta Toro Virus as monitored by electron microscopy

(4) Localization of virus-specific antigens in infected cells by immunofluorescences

(5) Analysis of the growth,concentration, and purification of virions.

To date, the major emphasis has been placed on protein characterization, virus morphogenesis and antigen localization, and for the most part with one virus, Punta Toro. This virus was initially selected for its rapid growth and plaquing characteristics. The rationale for concentrating initially on one virus was to avoid the possibility of cross contamination in the early stages of study, and to become familiar with the general properties of this group of viruses. Recently, studies have been initiated on the Chagres and Karimibad viruses.

This report will summarize the progress which has been made to date and the materials and methods which have been found most useful in the collection of these data. As this is the first annual report methodology will be detailed and will not be repeated in the renewal proposal. It will be shown in this report that, at least on an analytical scale, all of the experiments proposed in the initial research proposal regarding characterization of this group of viruses with respect to virus-coded proteins and virus-directed protein synthesis, antigen localization, and virion morphogenesis are within the sensitivity of currently available methodology. Although several sections of this proposal have been essentially completed it should be noted that this contract has been in effect for only five months, that technical assistance has been available for less than that period, and that CO_2 incubators which are necessary for some aspects of the proposed study have not yet been delivered.

Due to the large number of photographic presentations in this report, five complete sets of photographs have been submitted and less satisfactory replication have been included for the remainder of the required copies. If additional photographic copies are required they will be submitted as soon as possible.

III Morphology and Morphogenesis of Sandfly Fever Viruses (Punta Toro)

Electron microscopy (EM) of viruses or virus-infected cells suffers from sampling errors in that only a small percentage of any sample can be observed and also from the fact that it is often difficult to reconstruct the sequence or order of events from static EM images. Nonetheless, if properly controlled, electron microscopy can be very useful not only in determining particle morphology but also the events which occur intracellularly during virion morphogenesis. Such data are most useful when combined with biochemical data obtained from other procedures. If the same conclusions can be drawn from EM observations and from biochemically derived data, the chance of false conclusions is much reduced. As a complement to data which will be presented in later sections on the distribution of virus antigens in infected cells, and the synthesis of virus specific polypeptides, we have examined by electron microscopy the development of a sandfly fever virus (Punta Toro) as it occurs in our cell culture systems. Vero cells were chosen as a host cell as, in our hands, they consistently yield titers approaching 10^8 plaque forming units (Pfu)/ml. These cells were obtained from the American Type Culture Collection and were free of mycoplasma as detected by thin-section electron microscopy.

Methodology

Subconfluent monolayers in plastic flasks were infected with Punta Toro virus in Minimal Eagles Medium (MEM) with 2% fetal calf serum at a multiplicity of 5 Pfu per cell. Mock-infected cultures served as controls. At 15 hours and 35 hours after infection cells were scraped from the substrate plastic and fixed overnight at 4C in a solution of 5% acrolein and 0.25% glutaraldehyde (Polyscience, Warrington, Pa.) prepared in 50 mM sodium cacodylate buffer, pH 7.3. These cells were washed in the cacodylate buffer by centrifugation, and postfixed for 1 hour at room temperature in 1% osmium tetroxide (in 50 mM cacodylate) containing 1% tryptone and 0.5% NaCl. Cell pellets were embedded in 2% noble agar, stained in 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon 812 by the procedure of Luft (2). Ultra-thin sections were collected on carbon-coated colloidion grids, stained with uranyl acetate and lead citrate, and viewed in a Siemens Elmiskop 1A.

Results

By 15 hours after infection virus particles could be seen in virtually all cells. All virus particles were contained within, or budding into, smooth membrane vesicles (Figure 1 and 2), membrane structures with bound ribosomes were devoid of virions (Figure 2). Budding occurred predominantly in, but was not restricted to, the perinuclear areas (Fig. 2), corresponding to the area of maximal concentration of virus-specific antigens as seen by immunofluorescence (see below). Budding was never observed at the plasma membrane. Compared to uninfected cells, the smooth membranes appeared to be highly proliferated in areas where budding was observed. In other studies with Bunyaviridae it has been assumed that these membranes were derived from Golgi structures (3, 4), and this is consistent with our observations.

In many cells the cytoplasm contained numerous membrane-bound sacs (Fig. 2), which, as they were studded with ribosomes, were assumed to be distended endoplas- mic reticulum, although occasionally similar structures were seen as evaginations of the outer nuclear membrane (Fig. 3). Particulate nucleocapsids, as seen in alphavirus or flavivirus-infected cells (5, 6), were not seen which is consistent with the presumed helical symmetry of the phlebotomus group viruses (3, 7). Virions were seen in all stages of maturation (Fig. 4). In budding figures which were sectioned such that the bilayer nature of the membrane was evident, an increased electron density was apparent on the cytoplasmic side, presumably corresponding to viral ribonucleoprotein (RNP), and also an increased electron density on the lumen side which is presumably due to inserted viral glycoproteins (Fig. 1). Budding proceeds until a full-sized virion is formed at which time membranes at the base of the developing bud fuse, and the virion appears morphologically complete and residing within the cytoplasmic vesicle. The structures which are assumed to be viral glycoproteins are then on the outside of the virion forming spikes, and the structure believed to be viral RNP are on the inside forming the nucleocapsid (Fig. 1).

Intravesicular particles (Fig. 5) are round or oval in cross section with particle diameters which vary from 90 to 115 NM. Within the viral envelope the electron dense RNP is usually located at the periphery, directly beneath the envelope, leaving a relatively electron lucent center. Attempts to discern morphological differences between intra- and extra-cellular particles gave incon- sistent results, and it is assumed that virions are morphologically mature when budding has been completed.

Figure 1 shows that the viral RNP and the viral spikes can only be demon- strated on that part of the vesicular membrane which is directly involved in the budding process, and are absent on adjacent areas of host membrane. Also these internal and external membrane modifications appear to occur coincidently insofar as it was not possible to show viral RNP associated with a membrane area without spikes on the controlateral side. This suggests that viral RNP can attach only to membrane which has previously been modified with viral glycoproteins and pre- supposes some form of transmembranyl recognition, most likely mediated by a transmembranyl viral glycoprotein. A similar recognition is believed to occur between viral glycoprotein(s) and nucleocapsids of Sindbis virus (8, 9) and between viral glycoprotein and the M protein of several negative-stranded, helical RNA viruses (10), all of which bud from the plasma membrane.

Release of virus from infected cells apparently occurs by exocytosis (fusion of virus-containing vesicles with the plasma membrane). This was often observed at 15 hours after infection. In figure 6, a virus particle, having apparently been released from one cell, is trapped at the plasma membrane of an adjacent cell. In figure 6a, a virus-containing vesicle is observed at the junction of two cells, apparently in the initial stages of fusion with the plasma membrane. Pinocytotic vesicles (endocytosis) with or without virions were also frequently seen (Fig. 6b). These vesicles are distinguished from those described above in that they manifest a coat of "bristles" or "knobs" on their cytoplasmic face known to be characteris- tic of pinocytotic vesicles (11). One consequence of exocytosis to disease patho- genesis is that infected cells would likely contain virus antigens on their surface plasma membranes, despite the lack of surface budding, which would presumably render them identifiable to the immune system and hence to both antibody/complement- and cell-mediated lysis.

Thin sections from cells infected for 35 hours showed considerably fewer virus particles and budding particles were rarely seen. Apparently, under these conditions, the assembly of virions ceases prior to this time and virions do not accumulate within infected cells. Although many of the cells have released from the substrate plastic by this time, surface and nuclear membranes are usually found to be intact. Cytopathic effect (CPE) is, however, evident in that mitochondria are swollen and distorted and an electron-dense material is deposited on the cytoplasmic face of many cytoplasmic vesicles (Fig. 7). The nature of this material is unclear although its staining characteristics suggest that it may consist of disaggregating ribosomes or perhaps viral ribonucleoprotein, which although bound to membranes is unable to initiate budding. Round or irregular masses with similar staining characteristics are also found free in the cytoplasm (Fig. 8). Similar granular structures have been described by Murphy et al. (3) although it is unclear if these structures and those seen here are comparable. If these structures represent disaggregating ribosomes, other ribosomes must be still functional at 35 hours after infection as such cells continue to synthesize protein which is mainly virus-directed (see below).

Negative Staining

To determine the fine structure of extracellular particles, negative stains using 2% phosphotungstic acid and the procedure of Anderson (12) have been attempted. As unconcentrated media contains insufficient numbers of particles and the virus is apparently unstable in the density gradients used to date (see Discussion in Section IV), virus was concentrated from the media of infected cultures by immunoprecipitation. Dilutions of hyperimmune mouse ascitic fluid (obtained as a gift from Dr. Walter Brandt, WRAIR) were adsorbed twice with uninfected vero cells at 4C and added to clarified media supernatants (10,000Xg for 20 minutes). The mixture was incubated at 4C for 8 hours and then centrifuged at slow speed inaBrinkman microfuge (12,000 RPM) for 10 minutes. The pellet was taken up directly in 2% phosphotungstic acid, pH 7.2, and applied to carbon-coated colloidion grids. Aggregations of virus particles were readily observed by this procedure substantially free of host cell debris (Fig. 9). Although this procedure establishes an identity, or at least a cross-reaction between the virus used in these experiments and the virus used for immunization, and also provides an alternative to gradient centrifugation for virion purification which will be useful for biochemical assays (see Section IV), definitive fine structure studies could not be performed as virions were consistently positively stained by this procedure. Positive staining was also obtained when uranyl acetate was used as a contrast material. Positive staining was not due to the presence of antibody as media centrifuged at 100,000 xg in a Beckman ultra-centrifuge for 2 hours to pellet virions (and host debris) also yielded positively stained virions, presumably the clumping of virions in this case is due to the high speed pelleting procedure (Fig. 10). Additional studies are in progress with virions purified on glycerol and percoll gradients as well as with contrast media prepared at different pH values.

Discussion

Although significant cytopathic effect is not observed until 25-30 hours post infection in this system, morphogenesis of the virus clearly is occurring much earlier. The times chosen for thin-sectioning were based on early experiments in which maximal yields were obtained at 48-72 hours. In more recent

experiments maximal yields are obtained much earlier and we suspect that the virus is adapting to our cells. We now know that virus-directed protein synthesis can be detected without concentration procedures as early as 5 hours post-infection - which is as early as we have studied. Earlier times are now being studied by electron microscopy as well as with other procedures. However, from the data presented several conclusions can be made. 1) Virus morphogenesis occurs exclusively on smooth internal membranes and predominantly in perinuclear areas. 2) No particulate structures identifiable as virus components are seen in nuclei. 3) No nucleocapsids are visable prior to the time that viral ribonucleoprotein (RNP) condenses in the actual virus bud. 4) During times of maximal budding, viral RNP condenses on the cytoplasmic side of membranes only in areas in which a membrane thickening (presumed to be viral spikes) exist on the vesicular side of the membrane. 5) Neither structure is apparent on membranes adjacent to obviously budding particles. 6) The end result of virus budding is that particles (which appear identical to extracellular virions) became enclosed within vesicles. 7) These vesicles release virions by exocytosis at the plasma membrane.

Although it is clearly difficult to distinguish between virus escaping via exocytosis and virus being engulfed by pinocytosis, it is reasonable to assume the former occurs as: 1) virus was never observed budding from the plasma membrane despite screening several hundred infected cells, and 2) large numbers of virus particles can be seen on the outside surface of infected cells in the absence of observable cell lysis. Whether virus antigens exists on the surface of infected cells either as a function of vesicle fusion with the plasma membrane or of some other process is not yet known. However, this question will be addressed by ferritin-tagged antibody procedures as well as by membrane immunofluorescence.

These observations on virion morphogenesis are in general agreement with the conclusions of Murphy et al. (3) and Holmes (4), and provide more information as to the sequence of events occurring during budding. In addition, the timing of these events, as it occurs in our system, has been partially established which was not possible in Murphy's study as he studied infected animal tissues, or from Holmes' studies in which, for unknown reasons, samples were not harvested until 4 to 6 days post-infection. Thin-section analysis will also be carried out on infected phlebotomus cell cultures when such lines can be initiated.

IV.   Immunofluorescence and Light Microscopy of Infected Cells

Methodology and Results

To ascertain the effect of virus infection on cell morphology at the light microscope level and to allow indirect immunofluorescent studies on infected cells, vero cells were grown and infected on microscope studies designed for this purpose (Lab TEK). Such studies clearly do not allow conclusions to be drawn regarding virus morphogenesis, however, gross cell morphology may be observed without the sampling error inherent in electron microscopy, and by immunofluorescence the distribution of viral antigens in infected cells may be determined. Each slide contained four independent chambers which were infected with three dilutions of virus giving multiplicities of 10, 1 and 0.1. The fourth remained uninfected as a negative control. At 24 hours after infection

individual slides were processed for: 1) giemsa, 2) hematoxylin-eosin, 3) acridine orange fluorochrome and 4) immunofluorescent staining. The results from the first three procedures were similar and consistent and will be discussed together (data not presented). At 24 hours after infection all three procedures showed that the nuclei of infected cells are enlarged and the very prominent nucleoli present in uninfected cells are absent. The condensed chromosomes of mitotic figures which are easily observed by their intense yellow fluorescence with acridine orange are also totally absent in infected cells. Although the contribution, if any, of the nucleus to the replication of phlebotomus group virusus is unknown (see renewal proposal for discussion), it is clear that substantial alterations occur in the nuclei of infected cells.

Immunofluorescence was carried out by utilizing an indirect procedure with mouse hyperimmune ascitic fluid (obtained as a gift from Dr. Walter Brandt, WRAIR) which was adsorbed twice with uninfected vero cells, and the fluorescein-conjugated IgG fraction of rabbit anti-mouse gamma globulin. The primary reagent was prepared by the injection of infected mouse brain homogenates and should contain activity against all virus-coded proteins. Infected and uninfected cultures were washed with phosphate-buffered saline, pH 7.2 (PBS), air dried and fixed in acetone for 10 minutes at room temperature. A 1:10 dilution of the ascitic fluid in PBS was flooded over the cells and allowed to stand for 30 minutes at room temperature in a humid atmosphere. The cultures were subsequently washed extensively with PBS and reacted with the fluorescein conjugate for 30 minutes which was diluted 1:10 in PBS containing 0.1% Evan's blue used here as a contrast fluorochrome. The cultures were again washed, mounted in PBS containing 20% glycerol, and viewed in a Zeiss microscope operating in epifluorescence mode. By these procedures uninfected cells yielded only the dull red fluorescence of Evan's blue and were not stained by the fluorescein conjugate (Fig. 11).

At 24 hours, infected cells showed positive fluorescence throughout the cytoplasm, but a definite concentration of antigens was observed encircling the nucleus (Figs. 12-14). This perinuclear fluorescence differs from that seen in secretory cells (e.g. thyroid or pancreas) where secretory protein antigens are usually seen only to one side of the nucleus presumably corresponding to areas of Golgi concentration (13). It is also of interest that the Evan's blue fluorescence was often very intense in perinuclear areas and was usually surrounded by strong immunofluorescence (Fig 13). This increase in Evan's blue staining over uninfected control cultures is most probably due to an increase in glycoprotein synthesis (presumably viral glycoproteins) as Evan's blue is believed to show a specificity for glycoproteins (14, 15).

One of the purposes of determining the distribution of viral antigens by immunofluorescence was to be confident that in parallel experiments (in which this same antiserum is being used to precipitate labelled proteins for gel analysis) the nuclear fraction could be discarded without loss of viral specific proteins. Although most cells exhibited only a punctate fluorescence over nuclei, which could be explained by cytoplasmic inclusion overlaying the nucleus, this was not always the case (Fig. 12, 13, 14). In many cells fluorescent inclusion bodies were seen in the nucleus, with the same shape as the nucleus, and surrounded by a clear area. Such inclusions were never seen in uninfected controls. In conjunction with other data (described above) showing changes occurring as a function of

infection, and from thin sections which show an intact nuclear membrane at even later times, it is possible that some viral antigens are present in the nuclei (see Discussion below). Experiments to evaluate plasma membrane fluorescence on live and glutaraldehyde fixed cells, and whole-cell fluorescence at different times after infection are in progress.

Discussion

The hyperimmune ascitic fluid shows high activity in immunofluorescent assays and specificity insofar as uninfected cells are not labelled, host debris is not precipitated in immune-electron microscopy and labelled vero cell proteins are not precipitated in triton-X 100/DOC cell lysates (See below). Although this antisera has been adsorbed, only live vero cells have been used, a procedure which presumably would remove only surface host proteins. Antigens have been localized throughout the cytoplasm and perhaps in the nucleus with a definite perinuclear concentration. Various other light microscopy procedures have shown abnormalities in infected cell nuclei, but it is unknown if these are a direct or indirect consequence of virus infection. With regard to the role played by the nuclei in virus multiplication, it should be noted that in one study (16) with California encephalitis (a bunyavirus), actinomycin D at a concentration of 1 µg/ml inhibited virus yields by 97%. In the same study cell enucleation reduced yields by one and one-half logs and not all cells were successfully enucleated. Although other studies have shown an actinomycin D insensitivity (7) it should be noted that: 1) not all cells efficiently transport actinomycin D and are only poorly inhibited even at high concentrations (5 µg/ml) such as the vero cells used in this laboratory, and also 2) that influenza virus with a proven requirement for host cell transcription is only sensitive to this inhibitor at very early times (17). Therefore, I feel that the role played by the nuclei either directly (transcription of host genes) or indirectly (site of some virus function) remains an open question. These questions are best explored through the use of trioxalen (psoralen) derivatives and DRB inhibition and by isolation of nuclei (see renewal proposal).

V.   Analysis of Virion Structual Proteins and Intracellular Protein Synthesis

General Introduction

The ability to distinguish virus-coded proteins from host-cell specific proteins presupposes either the ability to purify virions from host cell components or the ability to recognize virus-specific proteins in whole cell lysates. Each of these approaches has advantages and disadvantages. The former would discern only virion structural proteins which conceivably, as is the case with vesicular stomatitis virus, could represent all of the primary products   coded for by the virus genome. There are no non structural proteins synthesized by VSV and no post-translational cleavages (10, 18). The latter approach, that of analyzing labelled cell lysates, allows the identification not only of virion structural proteins, but also non-structural proteins and precursor proteins, should they exist. The technical problems inherent in this approach relate to the difficulty in assigning a particular polypeptide to either host or viral origin. Even if virus replication results in an inhibition

of cellular messenger RNA synthesis and all cells can be infected, the residual activity of "long-lived" messenger RNA's often continues throughout the virus replication cycle. Comparing the profiles of infected and uninfected cells is useful, but not rigorous, as the host contains hundreds of proteins with every conceivable molecular weight and isoelectric point.

The use of DNA-specific transcription inhibitors has been useful in many virus systems, but assumes transport by the host cell and relative resistance of virus-directed transcription and still suffers from the inherent problem of long-lived host messengers. The ability to detect precursor polypeptides depends upon the relative rate of synthesis vs. processing. If detectable levels of virus protein precursors accumulate during a very short pulse and processing is delayed, pulse-chase experiments will identify such precursors. If such is not the case, processing must be inhibited by amino acid analogues, protease inhibitors, or through the use of temperature-sensitive mutants. Pulse-chase experiments have been useful in demonstrating precursor relationships in picornavirus-infected cells (19), but less so in alphavirus-infected cells (6). It should also be noted that in both of these systems approximately 10-100 fold more particles are produced than is the case with the phlebotomus agents.

An additional procedure useful in the concentration and identification of virus-specific proteins is immune selection, accomplished by various methods of immune precipitation or via solid phase immunoabsorbents or immune-affinity chromatography. This approach assumes serum activity against all virus-specific proteins (antigens), lack of activity against host cell components, and the ability to remove proteins associated non-specifically with immune complexes. Additional problems associated with immune selection procedures relate to the inherent insolubility of membrane glycoproteins in cell or virus lysates, and to the problems of separating antigens from immune complexes prior to analysis. The most reliable information is generally obtained by comparing the data obtained from several of these methods, run concurrently, and this has been carried out using the phlebotomus agents, Punta Toro and Karimibad.

It is useful to examine briefly what would be expected on the basis of the knowledge presently existing on this group of viruses. All of the Bunyaviridae which have been described contain a negative-stranded genome composed of three segments. The negative-stranded genome presupposes a virion-associated transcriptase which in all other negative-stranded viruses is a large polypeptide in excess of 150,000 daltons. As determined by Robeson, et al. (20), the molecular weight of these three segments for Punta Toro virus is 5.3×10^6 daltons. If a 10:1 coding capacity is assumed (molecular weight of RNA: molecular weight of protein), a theoretical figure which in fact is realized by Rhabdoviruses, influenza viruses, parainfluenza viruses, and reoviruses (21-24), Punta Toro RNA should code for a total of approximately 530,000 daltons of protein. As will be shown there is a minimum of four (and probably more) virus-coded proteins. Therefore, either one or more of these segments is transcribed into polycistronic mRNA's, the translation product of which is post-translationally cleaved, or multiple, monocistronic messengers are transcribed from single genome strands. Therefore, in addition to the structural proteins, cell lysates may very well contain precursor proteins. The existence of an envelope also indicates that at least one of the virion structural proteins will be a glycoprotein.

The data which will be presented are the results of experiments in which cell cultures have been infected with sandfly fever viruses and labelled with ^{3}H-leucine. Protein samples were then obtained from gradient fractions of concentrated extracellular virus, from cell lysate fractions, or from immune selection procedures, and were analyzed by discontinuous SDS gel-electorphoresis (PAGE) or by analytical gel isoelectric focusing (IEP). Radioactivity profiles were analyzed by fluorography.

Materials and Methods

Viruses, cells, media

Baby Hamster Kidney cells (BHK-clone 15) and cloned virus seeds were obtained from Dr. Joel Dahlrymple, WRAIR. Virus cultures were plaque-purified and stock cultures prepared in either Vero or BHK cells. Vero cells were obtained from the American Type Culture Collection. Primary chicken fibroblast cultures were prepared from SPAFAS embryonated eggs by standard procedures. All cell lines were maintained in Eagle's Minimal media (MEM) supplemented with 10% fetal calf serum.

Polyacrylamide Gel Electrophoresis

Discontinuous gel electrophoresis was carried out in a 28 cm slab gel apparatus (Bio-Rad Laboratories, Richmond, California) using a discontinuous system modified from Laemmli (25). DATD (N, N'-dialytartardiamide) was used as a cross-linker rather than Bis-acrylamide due to its better resolution of glycoproteins. The ratio of acrylamide to DATD was 30:1.6, and the stacking gel and the resolving gel were 5% and 13%, respectively. The sample buffer contained 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 10% glycerol, 0.005 M phosphate buffer, pH 7.0 and 5-10 µM phenylmethylsulphonyl fluoride (PMSF) as a protease inhibitor. All reagents were purchased from Bio-Rad Laboratories. Samples were dissolved directly in sample buffer preheated to 100°C for 2 minutes. Electrophoresis was carried out using constant voltage (60-90v) for 10-15 hours. The gels were then fixed and stained with coomassie brilliant blue. Samples containing non-ionic detergents were precipitated with absolute ethanol (at -20°C) to remove the detergent.

Analytical Isoelectric Focusing

IEF was performed in polyacrylamide slab gels run in a horizontal mode on a LKB multiphor which was maintained at 8°C by a recirculating water bath. The gels were polymerized according to Winter (26), but contained in addition to acrylamide, Bis-acrylamide, and 5% ampholytes (pH 3.5-10), 2% triton X-100 (Sigma) and 6M urea (Schwartz-Mann, ultrapure). The detergent and urea were necessary for solubilization of glycoproteins. Recently, it has been found that the new ampholytes prepared by Pharmacia produce more stable pH gradients (reduction in cathodic drift) and hence, an increased pH range at the basic end. Samples were solubilized in 2% triton-X 100, 6 M urea, and 5% ampholytes with or without 2% mercaptoethanol. Samples containing appreciable amounts of salt (> 10 mM) were either precipitated with 10% TCA (4°C) or with absolute ethanol (-20°C), or dialyzed against 50 mM ammonium bicarbonate and lyophilized to dryness.

Fluorography

To allow autoradiographic presentation of Tritium-labelled gel profiles, gels were impregnated with PPO according to the procedure of Bonner and Lasky (27). Gels were then dried onto filter paper or cellophane and exposed to Kodak XR-1 film.

Immune Selection of Viral Proteins

Hyperimmune mouse ascitic fluid (HMAF) was obtained from Dr. Walter Brandt, WRAIR, which was raised against the sequential injections of infected mouse brain. This preparation was adsorbed with uninfected vero cells and should contain activity against all viral specified proteins. Infected cell lysates or viral pellets were dissolved in 0.5% triton X-100, 0.02 M tris buffer, pH 7.0, 0.05 M NaCl and 10 µM PMSF. Whole cells were incubated in this buffer for 15 minutes at 4°C, gently homogenized, and the nuclei removed by centrifugation. DOC was then added to a final concentration of 0.5%. Direct immunoprecipitation was performed by the addition of various dilutions of HMAF to assure equivalence point precipitation, and incubated overnight at 4°C in Brinkman microfuge tubes. Precipitates were then collected by centrifugation, washed in lysis buffer, and dissolved directly in PAGE sample buffer.

Immune selection has also been accomplished by the preparation of a solid phase immunoadsorbent. The proteins in unfractionated HMAF were covalently bound to a beaded agarose support (affi-gel 10, Bio Rad Laboratories) in 0.1 M acetate buffer, pH 5.7. Unreacted sites were saturated by incubation of this complex in 1.0 M glycine. This complex can then be used either directly as an immunoadsorbent or in immune-affinity chromatography. In either case the antigens are eluted by treatment with 4M KSCN in lysis buffer with or without detergents, and concentrated by ethanol precipitation.

The advantages of solid phase immune selection are the following:

(1) equivalence points need not be determined, which in addition differ among different viral proteins
(2) antigens are obtained free of bound immunoglobulin
(3) host proteins are less likely to be non-specifically trapped in the immune complex
(4) since only antigen binding is required rather than the formation of a precipitate, this procedure is much quicker (one hour) which reduces the problems of protease activity and also allows the identification of viral proteins present in minute quantities or early after infection when insufficient protein is available for precipitation.

This procedure is also superior to methods utilizing staph protein under A insofar as the removal of immunoglobulins from antigens allows isoelectric focusing of virion proteins which is not possible with bound immunoglobulins.

Results

Although many electrophoretic and isoelectric focusing experiments have been carried out in attempts to identify and characterize virus-specific proteins, much of the information from early experiments has been difficult to interpret due to residual host cell protein synthesis. We have recently developed procedures in which the virus proteins in virus concentrates and in whole cell lysates can be specifically extracted and concentrated by immune selection procedures. The information that has been obtained by these procedures largely supplants that of earlier experiments. Therefore, the data from the earlier experiments will be summarized and that of recent experiments will be presented.

Three types of cell systems have been evaluated to date for their desirability in these experiments, vero, BHK-15, and primary chick embryo fibroblast (CEF) cultures. CEF cultures infected with Punta Toro virus exhibit some CPE within 24 hours; however, this CPE does not extend to all cells in the monolayer, and the yields of infectious virus are low (approximately 10^5 pfu/ml). We have not been able to demonstrate significant inhibition of cellular protein synthesis in infected cells, and the addition of actinomycin D (0.5 - 4 µg/ml) does not reduce host-directed protein synthesis to acceptable levels. We have, however, detected the labelled nucleocapsid protein of Punta Toro virus in media pellets from infected cells. It is our feeling that only a small proportion of the cells in these mixed cultures are susceptible to infection. Experiments with these cells have been discontinued. On the other hand, both BHK-15 and vero cell cultures are uniformly susceptible to infection, with round-cell CPE occurring synchronously in all cells by 24-36 hours after infection. Punta Toro virus-infected vero cells routinely produce $1-2 \times 10^8$ pfu/ml of culture media both in static or roller bottle cultures. The yields from BHK-15 cultures have been inconsistent, with yields in various experiments ranging from 10^6 - 10^8 pfu/ml. The reason for this variation is unclear. Initially, we had considered that the inability of vero cells to produce interferon might account for the generally higher yields obtained in these cells. However, if virus innocula are obtained by pelleting virions from media supernatants through a sucrose cushion, a procedure which would be expected to eliminate interferon, inconsistent yields are nonetheless obtained from BHK cells, relative to vero cells. We have also found that vero cells are highly susceptible to Karimibad and Chagres viruses, in which the cytopathic effect occurs 5-10 hours sooner than is seen with Punta Toro virus. Therefore, in our hands, vero cells have been found to be the most suitable line for these studies.

In order to identify virion structural proteins, clarified media supernatants from infected and ^{3}H-leucine-labelled vero cultures have been concentrated by Amicon ultrafiltration and subjected to equilibrium centrifugation in potassium tartrate gradients. The density of Punta Toro virus, as determined in separate experiments in which gradient fractions were monitored for infectivity is 1.165 g/cm^3. Therefore, in order to band virions in the center of the gradient a 5-35% (w/w) potassium tartrate gradient has been used. This gradient has been compared with a mixed glycerol-potassium tartrate gradient, 30% and 50% respectively, for their relative ability to separate virions from host cell debris. Both gradients were centrifuged to equilibrium

at 22,000 RPM for 12 hours in a Beckman SW27 rotor. These gradients were then
fractionated and their density profiles determined. Relevant fractions were
pooled, and the radioactive proteins precipitated in 10% TCA, and analyzed by
SDS gel electrophoresis. The results are presented in Fig. 16.

The fraction corresponding to a density of 1.165 g/cm^3 in the potassium
tartrate gradient is "D". This fraction contains three major proteins with
molecular weights of 66,000, 59,000, and 27,000 daltons. A fraction containing
these predominant proteins is also apparent in the glycerol/potassium tartrate
gradient. It has been shown in separate experiments that the 66 k and 59 k
polypeptides can be labelled with 2-^{3}H-mannose and thus these proteins are
believed to be virion glycoproteins. The 27 k protein is presumably
the major nucleocapsid protein. Although a relatively sharp peak of infec-
tivity has been obtained in other experiments, it is clear that viral proteins
are distributed throughout both gradients as are other labelled protein
species which are of presumed host origin. As these are equilibrium gradients,
it is not clear why viral proteins are not limited to more discrete areas of
the gradient; however, we have recently found that Punta Toro virus loses
infectivity very rapidly in potassium tartrate (3 logs in 12 hours at 4°C),
and hence the virus may be structurally unstable in this salt. However,
this virus is relatively stable in glycerol alone and such gradients are
presently being evaluated.

The infection of vero or BHK-15 cells results in a gradual suppression
of host cell protein synthesis, and an increase in virus-specific synthesis
(Fig. 17). In this experiment six identical BHK cultures were infected with
Punta Toro virus. At various times after infection, individual infected cul-
tures were treated with leucine-free media and labelled with ^{3}H-leucine
(50 µg/ml) for 5 hours at the times indicated. At the end of the labelling
period, cultures were washed, dissolved directly in SDS sample buffer, and
compared with uninfected cells by SDS-slab gel electrophoresis. The increasing
rate of synthesis of the nucleocapsid protein is visable in the coomassie
blue-stained profiles (left). The fluorograph (right) clearly shows the
diminution of host cell protein synthesis and an increasing rate of synthesis
of viral proteins. The nucleocapsid protein band is visible even at the earliest
time point (5 hours). At later time points the two higher molecular weight
proteins observed in gradient fractions are also visible. However, this exper-
iment illustrates that the assigning of specific polypeptides as either virus-
or cell-coded is not possible by this procedure alone, as host cell synthesis
is not markedly decreased during times of maximal viral protein synthesis and
continues even at times when viral synthesis is declining. It is also clear
that since these three putative viral proteins amount to only one third of the
theoretical coding capacity of the Punta Toro genome (7), several other virus-
specific proteins are probably obscured by the host background. Essentially
identical results to those shown here for BHK cells have been obtained from
infected vero cells.

We have also been unable to eliminate the host background protein synthesis
by the use of actinomycin D (AD) at concentrations ranging from 0.5 - 8 µg/ml.
Although the TCA-precipitable radioactivity incorporated into AD-treated cells
is reduced (50-70%), most host bands are still detectable by fluorography.
This is equivalent to the reduction seen in Sindbis virus-infected vero or BHK
cells treated with actinomycin D. However, the lower rate of
Punta Toro virus protein synthesis makes this background synthesis a corres-

pondingly greater problem. In several experiments, infected BHK or vero cells were labelled with ^{3}H-leucine, in the presence or absence of actinomycin D, lysed with 2% triton X-100 and 6M urea, and the proteins separated by analytical isoelectric focusing on slab gels. Despite the higher resolution of protein species afforded by this technique as compared to SDS gel electrophoresis, only the nucleocapsid protein could be consistently identified as a distinct species emerging from the host background. As in SDS gels, the nucleocapsid protein could also be distinguished by coomassie staining. The isoelectric point of this protein is high (approximately 9.2) as would be expected of a protein which must complex with RNA (a highly-charged polyanion).

Similarly, pulse chase experiments have given inconclusive results. Infected cells at 15 hours after infection have been pulse-labelled for 10 or 25 minutes and then chased for 1.5 hours. Several protein species could be detected after the pulse but not after the chase, and vice versa, but it is unclear if they represent virus or host proteins. However, the nucleocapsid protein was clearly labelled during the 10 minute pulse, indicating that the rates of viral protein synthesis are sufficient to allow pulse-chase experiments to be performed with this system when the immune selection procedures described below are combined with this procedure.

Therefore, to decrease the interference of host cell-specific synthesis and to increase the sensitivity of detecting virus proteins, direct immunoprecipitation procedures were used. The hyperimmune mouse ascitic fluid (HMAF) used in these experiments was prepared against Punta Toro virus-infected mouse brain homogenates by Dr. Walter Brandt (WRAIR). Two 75 cm^2 flasks of vero cells were infected with Punta Toro virus at a multiplicity of infection of 5, mock-infected cultures served as controls. At 18 hours after infection the cultures were preincubated for one hour in leucine-free media containing dialyzed fetal calf serum, and subsequently labelled in this media with 50 microcuries/ml of ^{3}H-leucine for an additional 6 hours. Cytoplasmic lysates were prepared in 2.5 mls of lysis buffer as described in methods (above) and pre-centrifuged in a Brinkman microfuge. The media from infected and control cultures were clarified at 10,000 x g for 30 minutes and then centrifuged at 100,000 x g for 2 hours. Pellets were dissolved in 1 ml of lysis buffer. The HMAF was adsorbed twice with live vero cells at 4°C for 30 minutes and also pre-centrifuged. Various amounts of HMAF were then added to 0.5 ml aliquots of cell lysates or to 0.3 mls of the media pellet lysates and incubated overnight at 4°C. All reaction mixtures were then centrifuged in the Brinkman microfuge for 4 minutes, and the precipitates washed twice in lysis buffer before being dissolved in SDS sample buffer. TCA precipitable counts were then determined. The results are given below (Table 1) and the electrophoretic separation of precipitated protein is shown in Fig. 18.

TABLE 1

Sample	Amount HMAF added (microliters)	Total counts precipitated (CPMx10³)
infected cell lysate		
#1	0	0.14
#2	10	17.4
#3	100	923.0
#4	300	1,053.1
#5	500	266.7
uninfected cell lysate		
#1	0	4.2
#2	10	8.8
#3	300	2.1
infected media pellet		
#1	0	2.3
#2	20	738.9
#3	300	1,272.2
uninfected media pellet		
#1	0	24.9
#2	20	5.5
#3	300	4.7

The "Total counts precipitated" column header reads $CPMx10^3$.

In Table 1 it can be seen that a large number of counts was specifically precipitated from infected cell lysates and media pellets relative to uninfected controls and those samples in which HMAF was not added. It is also apparent that optimal concentrations of HMAF are critical for efficient precipitation, as would be expected of a direct immunoprecipitation, insofar as equivalence points must be achieved to avoid antigen or antibody excess.

From the SDS gel fluorograph shown in Fig. 2 it is apparent that:
1) The background of host protein synthesis seen in the unprecipitated whole cell lysate (sample #2) is not present in the immunoprecipitates.
2) The three proteins described in the previous experiments are present in high concentrations(66 k, 59 k, 27 k).
3) A very high molecular weight protein, in excess of 130,000 daltons, is seen at the top of the gel from samples obtained from infected cells and virus pellets.
4) Two additional protein species have been precipitated which migrate ahead of the nucleocapsid band. These proteins have also been seen in lysates of infected BHK and L cells and thus are believed to be virus-specific.
5) None of these proteins is present in control lysates or from infected-cell lysates to which no antibody was added.
6) These proteins have different equivalence points with respect to the amount of HMAF added. This is especially noted in the precipitation of the two glycoproteins relative to the nucleocapsid protein from the viral pellets.

Using this technique it will now be possible to repeat the pulse-chase experiments to determine if unstable precursors can be detected, and if so to follow their fate during the chase period. If such precursors cannot be detected directly, amino acid analogues and protease inhibitors will be used to inhibit cleavage.

It will not, however, be possible to utilize this methodology to select viral proteins for isoelectric focusing analysis since the conditions required to separate antigen-antibody complexes are imcompatible with this procedure. Therefore, solid phase immunoadsorbents have also been developed (as described above in Methods) in which antibodies have been covalently bound to agarose support beads. This technique will also be more convenient than direct immuno-precipitation, as has been demonstrated above, several dilutions of serum are required for each experiment to insure that all viral proteins are precipitated. The adsorption of Punta Toro virus antigens to such a solid phase support, and their subsequent elution in 4M KSCN buffer is described below.

Immunoglobulins in the HMAF used above were bound to affi-gel 10 as described in Methods. The infection, labelling, and lysis of Punta Toro virus-infected cells were as described above for direct immunoprecipitation except that the infected cells from two roller bottles were lysed in a total of 10 mls of lysis buffer. The cytoplasmic lysate was allowed to react with the immuno-adsorbent for 1 hour at $4^{\circ}C$ with gentle shaking. The immunoadsorbent was then transferred to a column and washed extensively with lysis buffer (without detergent). 4M KSCN was then added to the column in lysis buffer (without detergent) in 5 ml aliquots. The elution of bound antigens from the column is given in Table 2.

TABLE 2

Eluant	Total counts removed in 5 mls ($\times 10^6$)
lysis buffer alone (final wash)	
#1	0.1
#2	0.06
lysis buffer with 4M KSCN	
#1	1.9
#2	3.9
#3	0.71
#4	0.13
#5	0.04
#6	0.02
Total removed in KSCN	6.7
Total remaining on column	1.26
Efficiency of elution	84%

It can be seen in Table 2 that 6.7 million CPM of presumed Punta Toro virus protein were specifically bound to the immune adsorbent and specifically eluted by KSCN. Preliminary experiments have shown that these proteins give SDS gel profiles essentially identical to those shown in Fig. 18. The nature of the proteins which remained bound to the column has not been determined.

Direct immunoprecipitation has also been performed on cells infected with Karimibad virus, by procedures identical to those described for the direct immunoprecipitation of Punta Toro virus antigens. The results of the immunoprecipitation are tabulated in Table 3 and the electropherogram is shown in Fig. 19.

TABLE 3

Sample	Amount HMAF added (microliter)	Total counts precipitated (cpm x 10^3)
infected cell lysates		
#1	0	9.4
#2	10	34.6
#3	100	535.7
#4	300	719.9
#5	500	858.8
control cell lysates		
#1	0	29.4
#2	10	22.8
#3	100	15.4
#4	300	10.3
#5	500	13.0
infected media pellet		
#1	0	1.5
#2	10	1.5
#3	100	3.5
#4	300	21.8
control media pellet		
#1	0	0.09
#2	10	0.12
#3	100	0.14
#4	300	0.07

The results in Table 3 show that relatively large amounts of radioactive proteins are also precipitated from Karimibad virus-infected cells. These results differ somewhat from those obtained from Punta Toro virus-infected cells insofar as less radioactivity was precipitated from virus pellets and somewhat more was precipitated from uninfected controls. The electropherogram reflects these differences as well as demonstrates several more apparently virus-specific polypeptides. Twelve discrete polypeptide bands are seen in precipitates from infected cell lysates of which four have also been precipitated from uninfected cells. The molecular weights of these proteins have been tabulated in Table 4. It is not yet known which of these proteins are

glycoproteins although the band migrating with an apparent molecular weight of 60,000 daltons can be resolved into two bands if the fluorograms are exposed for short periods of time, and also if viral pellets are dissolved directly in SDS buffer and analyzed. It is not yet known whether these two bands represent heterogenous glycosylation of a single polypeptide or if, in fact, they represent two distinct species. A similar problem relating to the separation of these two proteins has recently been discussed by Robeson et al. (7). It should be noted that in this experiment and in related studies in which virus pellets were analyzed directly, and in the studies reported by Robeson et al. (7), only these two polypeptides and the apparent nucleocapsid band have been shown to be virus structural proteins. It is therefore not yet clear whether the other polypeptides demonstrated here, which are assumed to be virus-specific proteins, are non-structural proteins or structural proteins which are present in virions at below presently detectable levels. It is also not yet known whether these bands represent unique polypeptides or whether precursor-product relationships exist. As with Punta Toro virus these questions are presently under investigation using pulse-chase methodology with or without the use of amino acid analogues and protease inhibitors.

Conclusion

Despite the fact that the rates of viral protein synthesis which occur in sandfly fever virus-infected cells are relatively low and that the residual levels of host protein synthesis remain relatively high, the immune-selection procedures documented here should be able to identify virion structural proteins, non-structural proteins and unstable precursor proteins. In addition, cross-immunoprecipitation would allow for a very precise definition of the cross reactions which exist in this group of viruses. These methods will also allow the monitoring of experiments designed to characterize the mechanisms which control the synthesis of these proteins and their subcellular distribution in infected cells-experiments which have been suggested in the accompanying proposal. One additional point should be raised here. The several quantitative differences in the polypeptide profiles obtained with the two viruses used to date could be explained by differential rates of protein synthesis, comigration of polypeptides, or by differential reactivity of the antisera used. However, it should also be considered that since the grouping of sandfly fever viruses is based on serological relationships, many at the limits of detection, and since the basic virology of these viruses is only now beginning to be studied, it is possible, if not probable, that significant differences exist in the replication of these viruses which are presently grouped together.

TABLE 4

Apparent Molecular Weights of Punta Toro and Karimibad Virus Proteins

Band number	Punta Toro	Karimibad
1	138,800*	136,000
2	66,300	74,500
3	59,000	60,000
4	26,700	51,000
5	23,000	31,000
6	22,000	25,000
7		14,000
8		13,500

*approximate

Growth of Cells on Microcarrier Beads

In order to produce sufficient quantities of virus to allow preparative separation of virion antigens, preliminary experiments have been carried out with various microcarrier systems. Initial findings have been encouraging and it is felt that these procedures coupled with flat-bed isoelectric focusing will provide enough purified antigens to raise monospecific antibody (1978 proposal, page 23) and to use as experimental vaccines (see accompanying research proposal).

Microcarriers are small (150 μm) beads with diethyl-amino-ethyl (DEAE) or related groups at the surface. These charged compounds allow the attachment of surface-dependent cells to these beads and a monolayer eventually forms around each bead (see Fig. 15). Microcarrier cultures are maintained in spinner flasks in the same media used for static or roller bottle cultures. Media changes, trypsinizations, or virus infections are accomplished by allowing the cell-coated beads to settle into a small volume and removing the supernatant. Five-liter cultures are conveniently established in spinner flasks with 25 grams of microcarriers, and theoretically have a surface area equivalent to 300 roller bottles (35). However, the yields of cells obtained from these cultures are usually somewhat less than this figure would imply (see below), and not all cells grow efficiently on a given carrier type. Another practical problem is that cells do not readily transfer from one bead to another during logarithmic growth; hence, large numbers of cells are necessary as an innoculum if all beads are to be efficiently used. Nonetheless, these cultures offer significant advantages over conventional roller bottle cultures with respect to handling, cost, contamination control and uniformity.

There are three types of microcarrier beads which are commercially available. The microcarriers available from Flow Laboratories are crosslinked dextran beads with DEAE exchange groups attached. These are the original microcarriers developed by Levine (35). However, these beads are considerably more expensive than the other forms available, and we have not found any advantage in their use. The microcarriers from Pharmacia are also crosslinked dextran (Sephedex); those available from Bio-Rad Laboratories are polyacrylamide. The Pharmacia and Bio-Rad products have been tested for their suitability for producing large cultures of BHK-15 and vero cells. The relative yields of cells produced in these and other types of cultures are given below. Microcarrier cultures were established at a bead concentration of 5 grams per liter.

Culture	BHK-15	Vero
75 cm^2 flask (Corning)	2.0×10^7/culture	7.6×10^6/culture
490 cm^2 roller bottle (Corning)	8.75×10^7/culture	3.2×10^7/culture
Bio-Rad microcarriers	4.75×10^9/liter	1.5×10^9/liter
Pharmacia microcarriers	3.6×10^9/liter	1.0×10^9/liter

Although these cell counts were performed by hemacytometer methods and should therefore be considered approximate, it is clear that each liter of microcarrier culture produces a quantity of cells equivalent to 30 to 50 roller bottles. A five-liter culture, which can be established with little more difficulty than a standard roller bottle culture, therefore, contains the equivalent of between 150 and 250 roller bottles. In our hands the growth kinetics of cells on microcarriers is about 80% of that achieved in roller bottles. The Bio-Rad

microcarriers seem somewhat more efficient than the Pharmacia product for the growth of BHK-15 cells; however, their use with our line of vero cells results in extensive cross-bridging of beads which settle out of the stirred culture. Both of these bead forms can also be used to line the inside of standard roller bottles as the charge differential between the positively-charged beads and the negatively-charged glass results in a strong electrostatic binding. In this case the cells are removed by EDTA or trypsin in magnesium and calcium-free buffer, and the beads removed in Hanks' salt solution containing ten times the normal concentration of magnesium. This procedure is convenient for studies in which infected cells are to be labelled with radioactive precursors as there is an increase of five-fold in the surface area without a substantial increase in the amount of media required.

The microcarrier cultures are infected by allowing the beads to settle into a minimal volume of media to which virus innoculum is added, and the culture is slowly stirred. After one hour of adsorption, media (with 2% fetal calf serum) is returned to the original volume. In preliminary experiments we have observed that vero microcarrier cultures produce $5-8 \times 10^7$ pfu/ml, which on a per cell basis, is approximately the same yield obtained from static cultures. Although the particle to pfu ratio is not yet known, in other arbovirus systems it is usually 10 to 100. Therefore, the production of virus particles to obtain sifficient amounts of purified antigen is not expected to be an insurmountable problem, especially when the large size of these viruses is considered. These particles have about 8 times the mass of alphaviruses and 15 times the mass of flaviviruses. Although relative yields of various viruses differ when microcarrier and static cultures are compared (35), it is considered probable that microcarrier cultures would also be useful in the propagation of other arboviruses for which vaccines are desirable.

VII. Experiments to be Pursued or Completed During the Remainder of the First Contract Year

The data and methodology summarized above will serve as a basis for the following lines of experimental study, most of which can be realistically expected to be completed in the current contract year.

A. Electron Microscopy of Virus Development

Earlier and intermittent time points will be taken to allow a more precise definition of the timing and sequence of maturational events which occur during morphogenesis. These studies will also be expanded as follows:

(1) by the use of hyperimmune mouse ascitic fluid (HMAF) and ferritin-tagged rabbit anti-mouse IgG (Cappel Laboratories) to determine if viral antigens are present on the surface of infected cells.

(2) by the use of HMAF alone to determine if anti-virus immunoglobulin will prevent the exocytosis of virus - as it interferes with the release of other viruses which bud directly from the plasma membrane (28).

(3) by the use of tunicamycin, a potent and specific inhibitor of glycosylation, to determine if budding is inhibited in the absence of glycosylation. Although budding of Sindbis virus is inhibited by tunicamycin (30), it is thought that this relates to a defective transport of the unglycosylated proteins from the Golgi apparatus to the plasma membrane (29). As sandfly fever viruses are capable of budding directly into the Golgi apparatus, it would be of interest to determine if budding is also impeded in this system by this inhibitor. Conceivably, unglycosylated virions may be formed.

(4) by determining the effect on virion exocytosis of calcium-free media. Exocytosis, as it relates to the release of secretory proteins, has been studied in a variety of different cell and tissue systems. In each case, it has been shown that calcium is required, and that in the absence of this ion, golgi vesicles accumulate in the cytoplasm. Exocytosis resumes upon the re-introduction of calcium. If calcium exerts a similar effect in our virus-infected cells, the role of exocytosis in virus release could be more rigorously demonstrated. In addition, relatively large quantities of virus could conceivably be released from inhibited cells into relatively small volumes of calcium-containing media which would have practical advantages in virus concentration and purification (33).

B. Immunofluorescent Localization of Antigens

Additional time points will also be taken in these studies. Additionally, membrane fluorescence will be performed and the data correlated with the ferritin-tagging experiments noted above. Indirect immunofluorescent will also be performed on nuclei isolated by nonionic detergents and hence free of cytoplasmic debris (34). Combined with immunoprecipitation of labelled proteins from nuclei (see renewal proposal) the question as to whether viral antigens exist in the nuclei of infected cells should be answered.

C. The characterization of virion structural proteins and intracellular virus-directed protein synthesis will be extended to the other human pathogens in the sandfly fever group using the immune-selection procedures described above. Characterization will include both electrophoretic analysis and analytical isoelectric focusing, the latter necessary to design the preparative (flatbed) isoelectric focusing conditions for isolation of larger quantities of antigens. In addition, a thorough search will be made for non-structural or precursor proteins in Punta Toro and Karimibad virus-infected cells. If immune selection and concentration of antigens from infected and pulse-chased cultures do not reveal such proteins, attempts to inhibit cleavage will be made through the use of amino acid analogues, zinc ions, and tunicamycin - all of which have been useful in the analysis of other virus systems (19, 30). These studies are expected to continue into the second contract year and will be carried out in conjunction with related studies designed to elucidate the controls imposed upon virus gene expression (see Section III of renewal proposal). The ability to detect minute amounts of viral proteins through the use of solid phase immunoadsorbents will also enable studies to be performed in which the synthesis of viral proteins is monitored at various times after infection - including very early time points when virus-specific synthesis constitutes only a small percentage of the total protein translated. If the molecular weights of the RNA genome segments reported for Punta Toro virus (20) are accurate, it is highly likely that other proteins, as yet unrecognized, are synthesized in infected cells.

D. From the data presented above in this report it is clear that the relatively low-titered preparations of virus and the difficulty in obtained purified virions do not preclude the analysis of sandfly fever virus replication or of virus-coded proteins on an analytical level. However, this restriction will impede the progress of other experiments which will require large amounts of purified virus and antigens. Most notably the preparation of sufficient quantities of individual proteins necessary to obtain monospecific antisera and for the production of experimental immunogens would not be possible (see 1978 proposal, page 23, and renewal proposal, Section IV). Therefore, increasing emphasis will be devoted to studying the parameters which effect the yields obtained in cell cultures - cell type, multiplicity of infection, type of culture, and the influence of interferon will continue to be studied. At present it appears that the enormous number of cells which can be relatively easily and inexpensively grown on the various microcarrier systems which are now commercially available will greatly facilitate the production of large amounts of virus both in this and other arboviral systems.

The concentration and purification of particles free from host cell components is still a difficult problem. However, as it is now known that Punta Toro virus is very unstable in potassium tartrate it is clear that density gradients formed from this salt alone or mixed with glycerol represented a poor choice. The virus retains infectivity in glycerol alone and preliminary data using pure glycerol gradients indicate that better resolution is obtained in these gradients (40-85%). Percoll, an inert colloidal silica compound designed especially for gradient centrifugation by Pharmacia will also be assessed for its applicability to these viruses.

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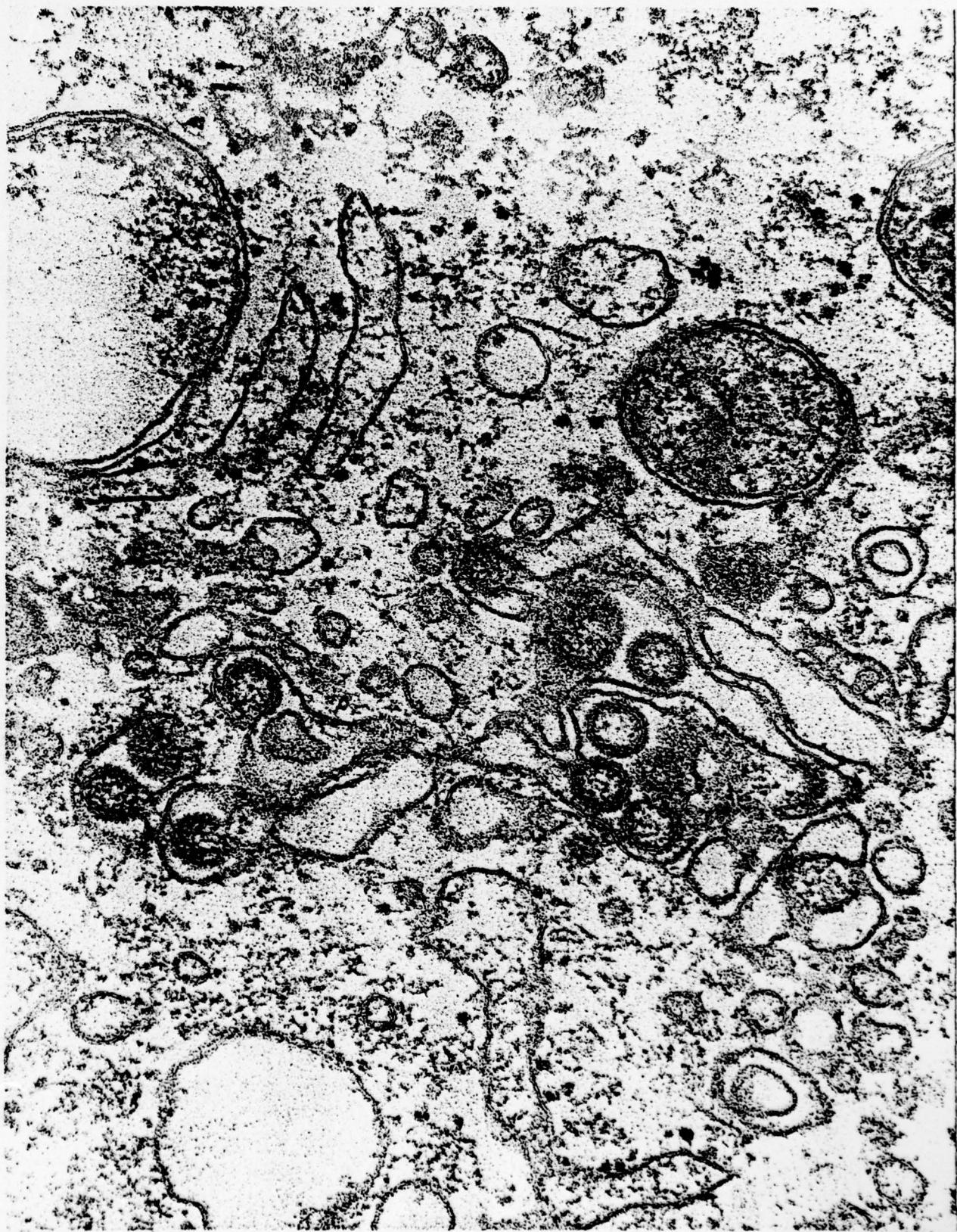
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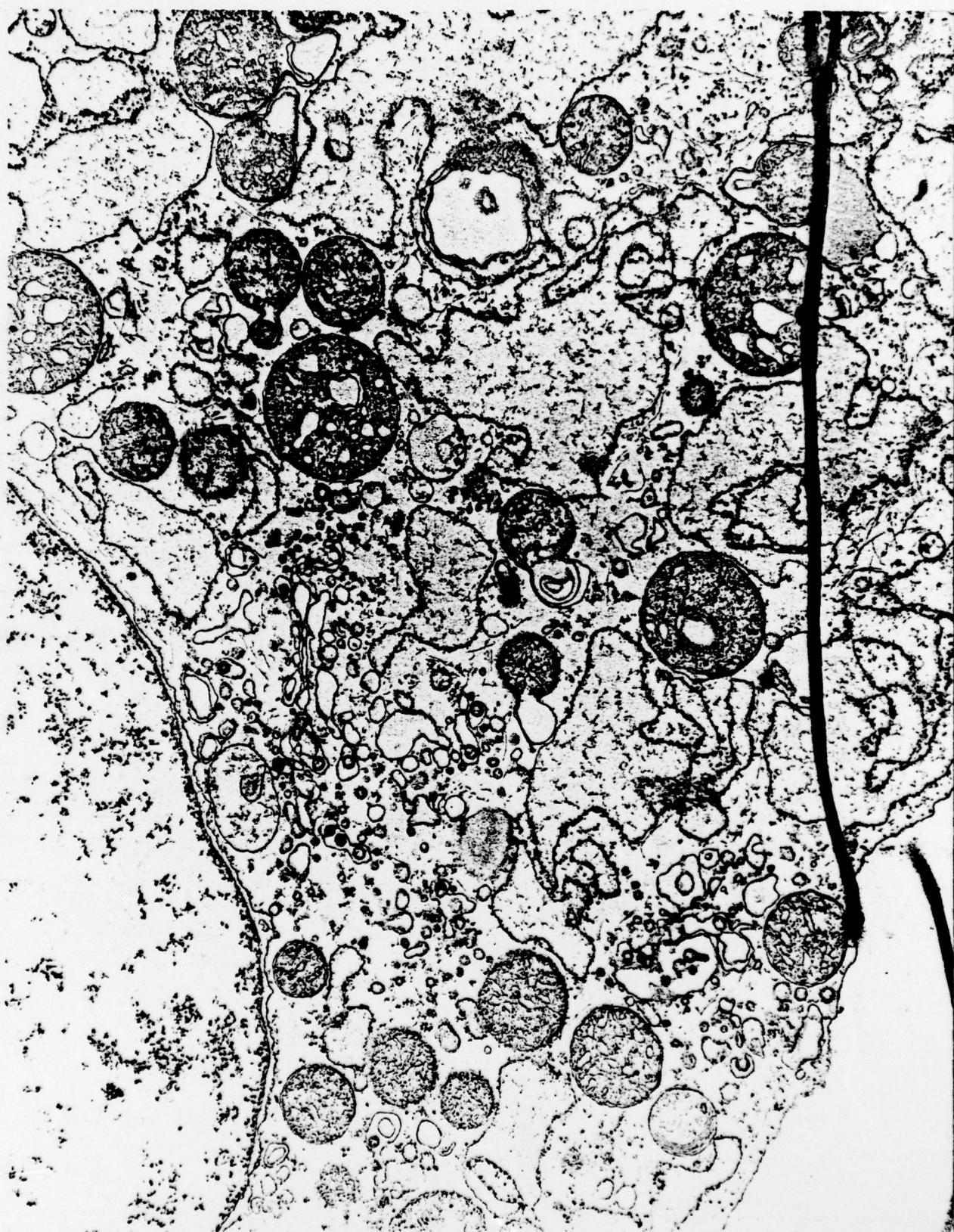
FIGURE 2

FIGURE 3


FIGURE 4
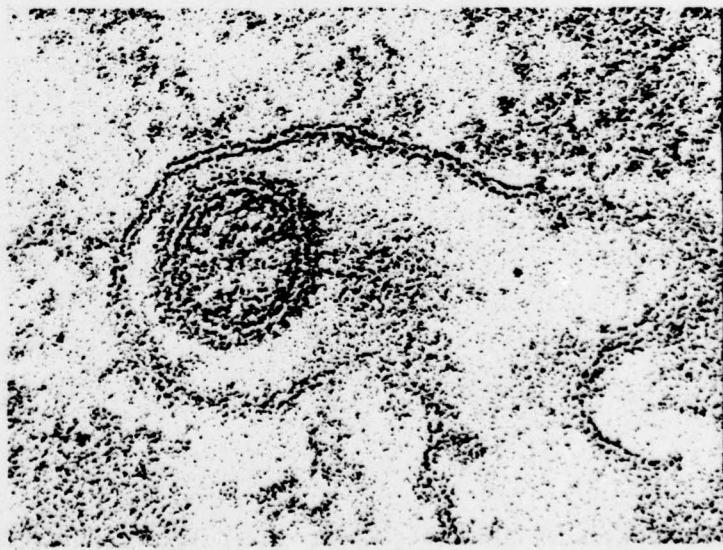

FIGURE 5

FIG. 6b

FIG. 6a
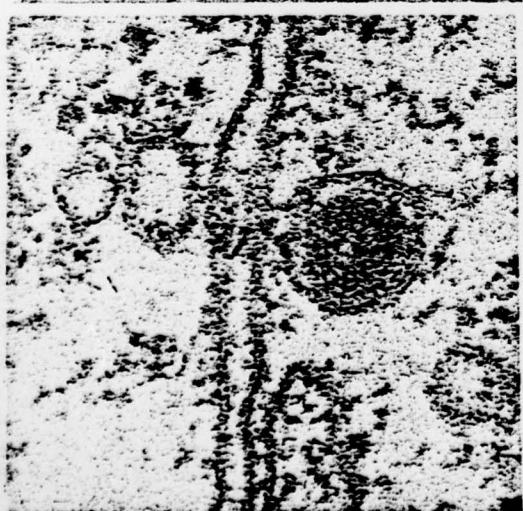
FIGURE 6

FIGURE 7
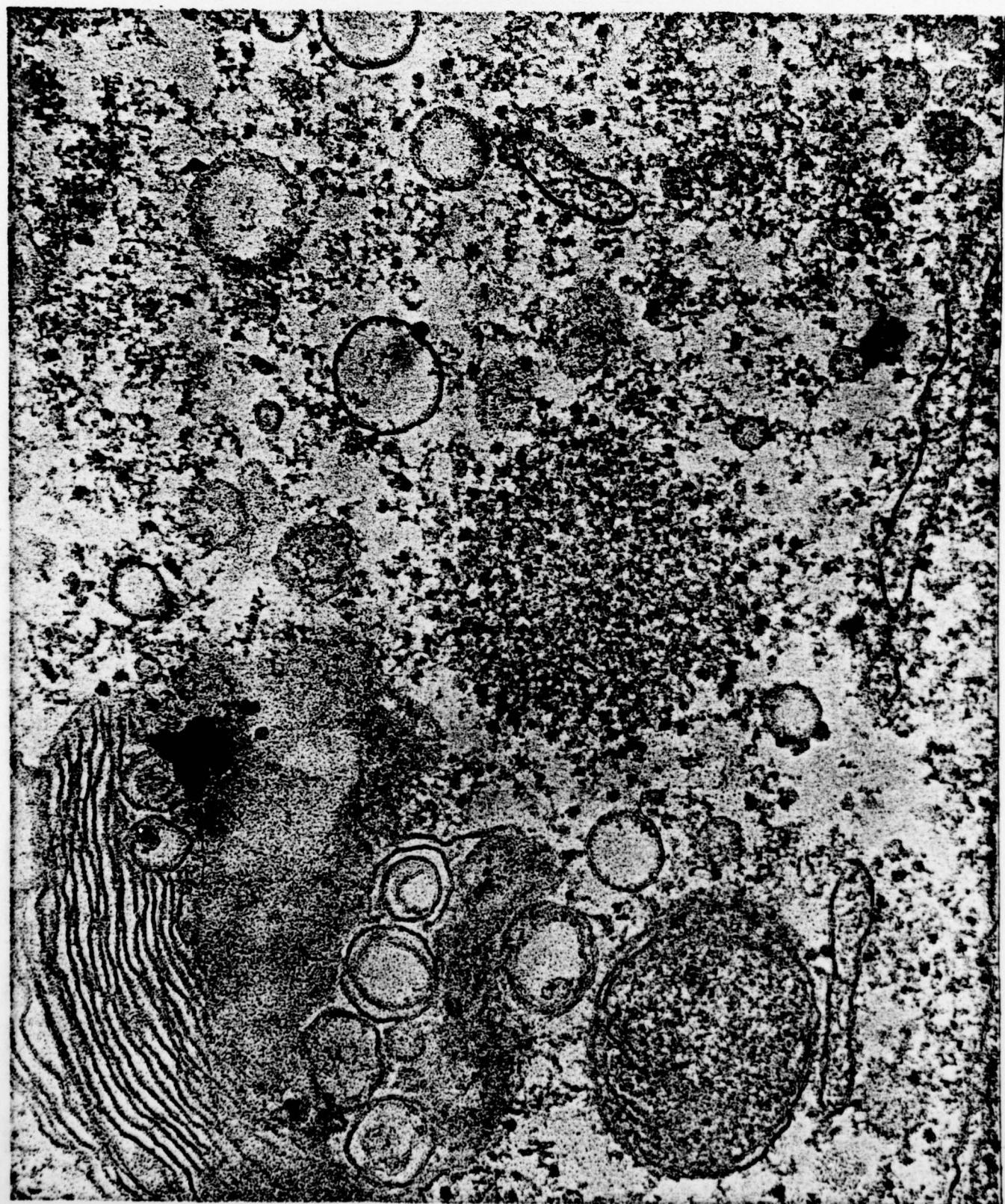
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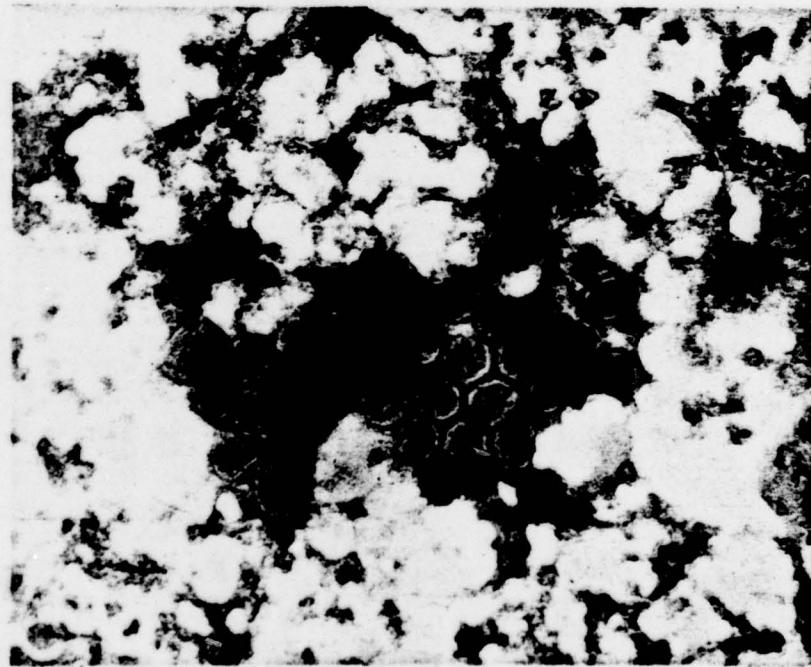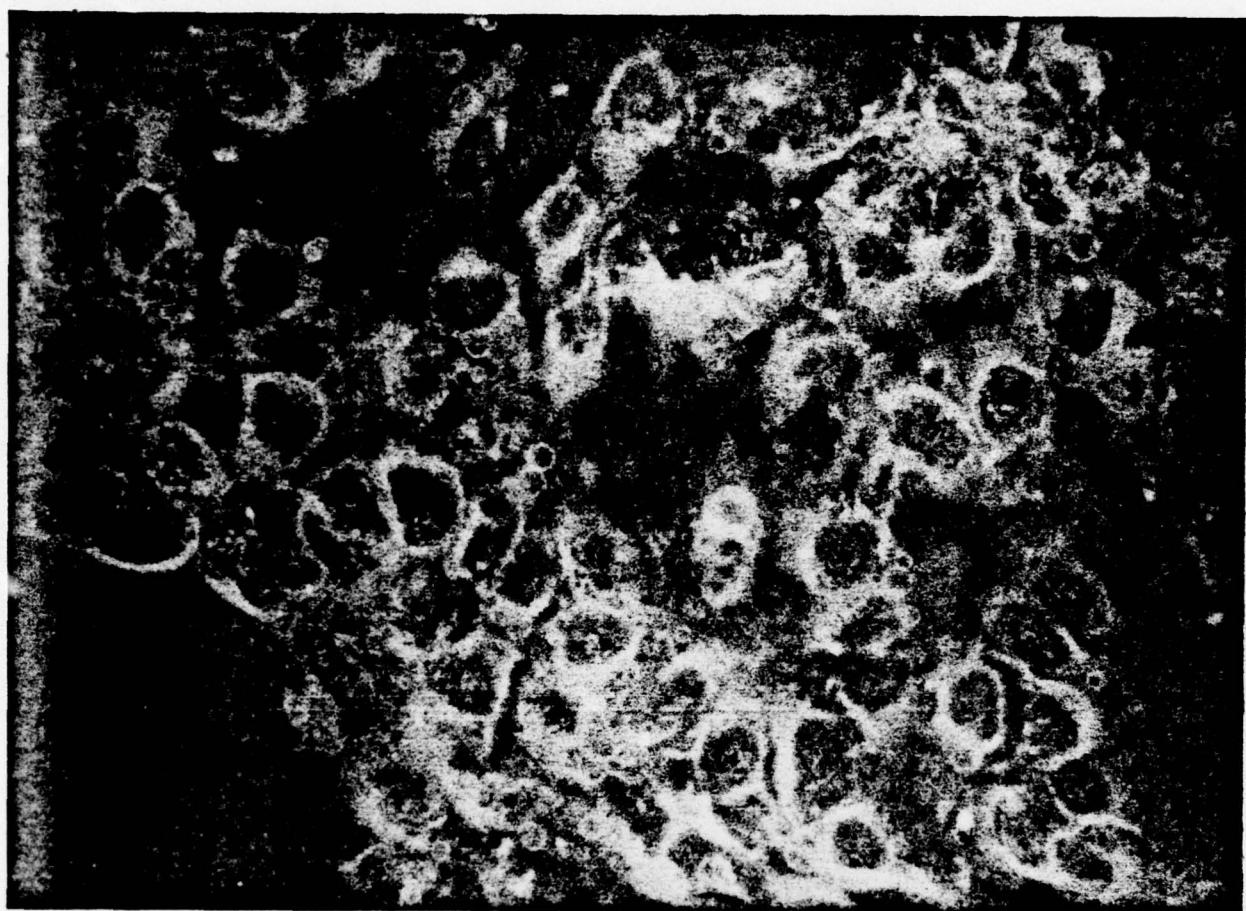
FIGURE 9

FIGURE 10

FIGURE 11
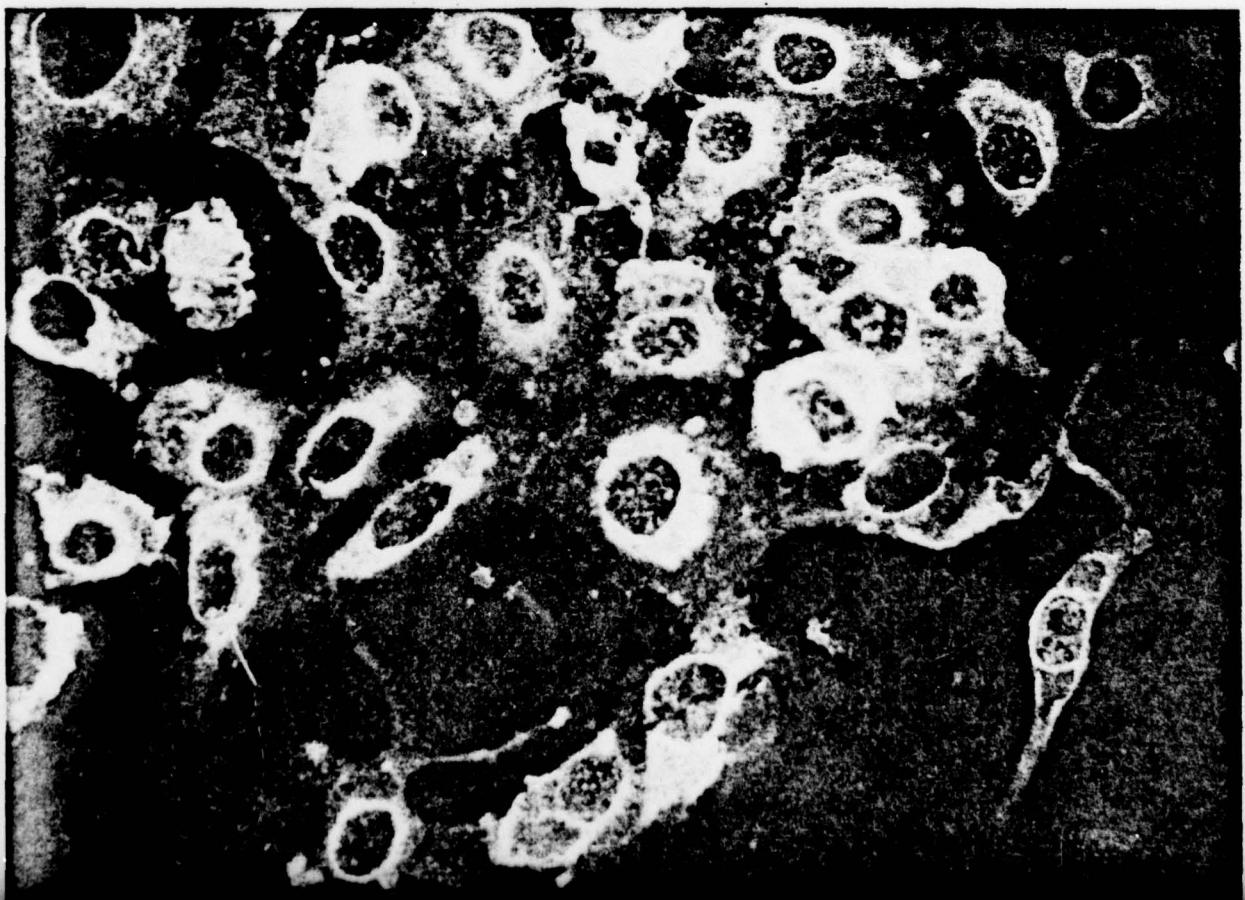
FIGURE 13

FIGURE 12
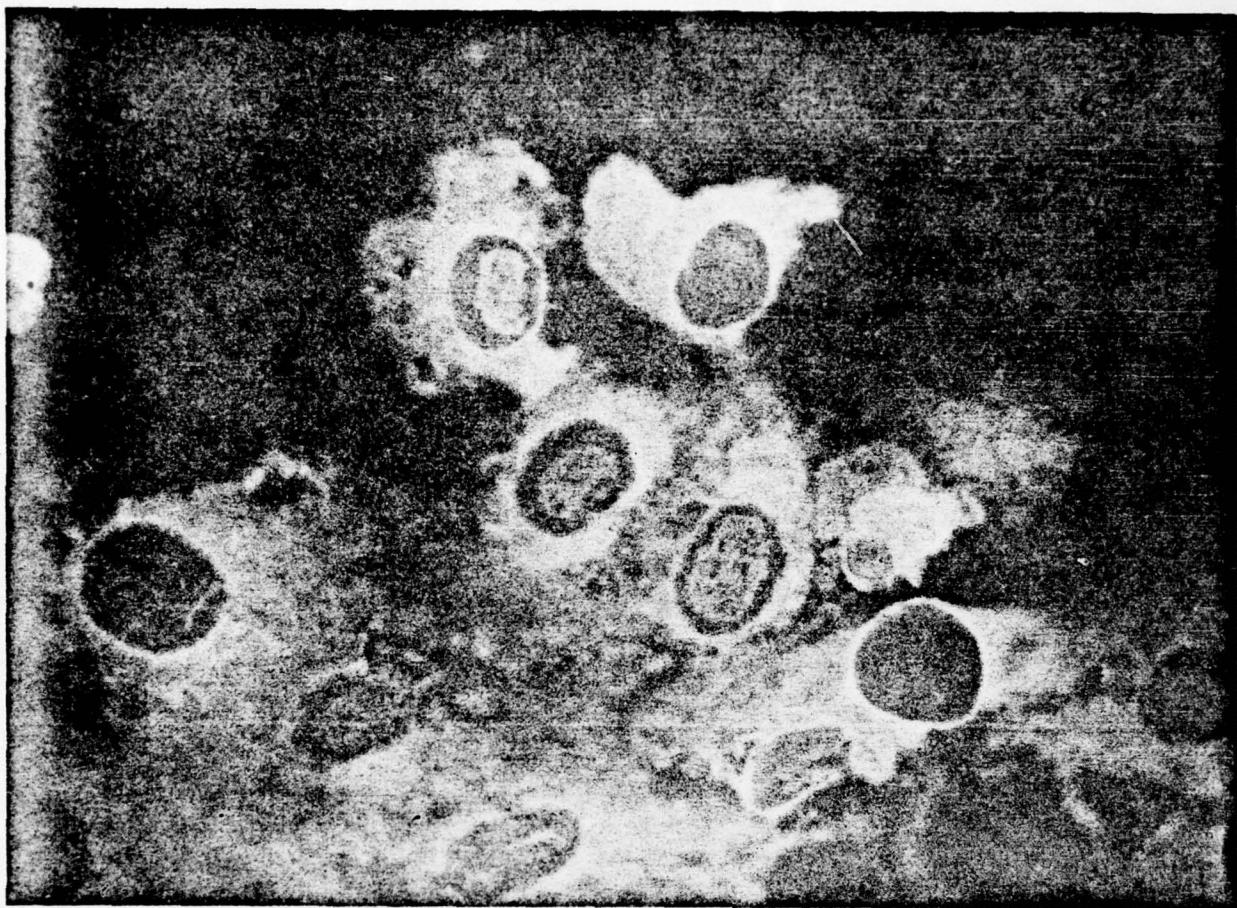
FIGURE 14

FIGURE 15

36
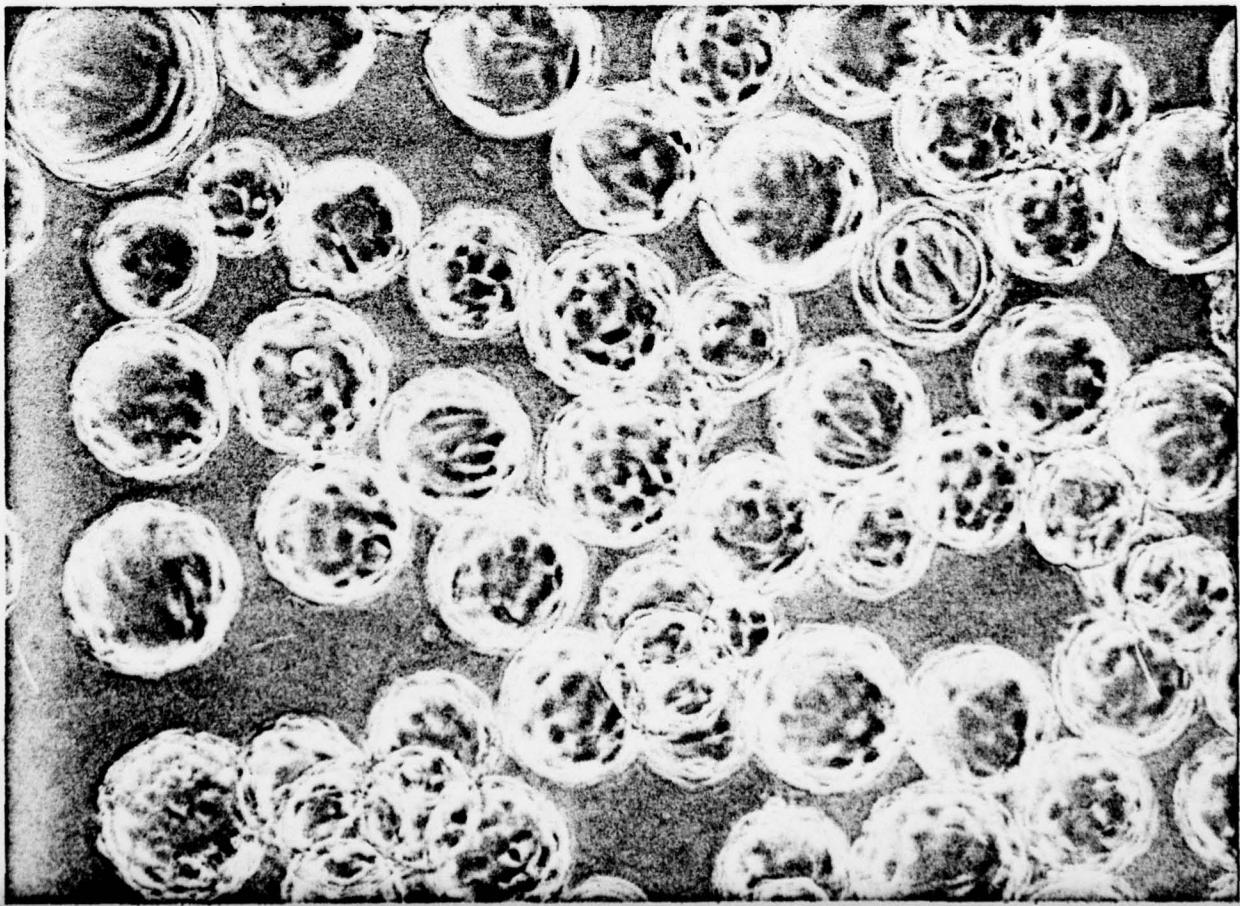
POOLED FRACTIONS   Figure 16

5 - 35% (w/w)   glycerol/potassium tartrate
potassium tartrate   (30%, 50% w/w)

Uninfected BHK

P.T.V. Inf. BHK- 5 hrs. p.i.

P.T.V. Inf. BHK- 9 hrs. p.i.

P.T.V. Inf. BHK-19 hrs. p.i.

P.T.V. Inf. BHK-24 hrs. p.i.

P.T.V. Inf. BHK-28 hrs. p.i.

P.T.V. Inf. BHK-43 hrs. p.i.

Uninfected BHK

P.T.V. Inf. BHK- 5 hrs. p.i.

P.T.V. Inf. BHK- 9 hrs. p.i.

P.T.V. Inf. BHK-19 hrs. p.i.

P.T.V. Inf. BHK-24 hrs. p.i.

P.T.V. Inf. BHK-28 hrs. p.i.

P.T.V. Inf. BHK-43 hrs. p.i.
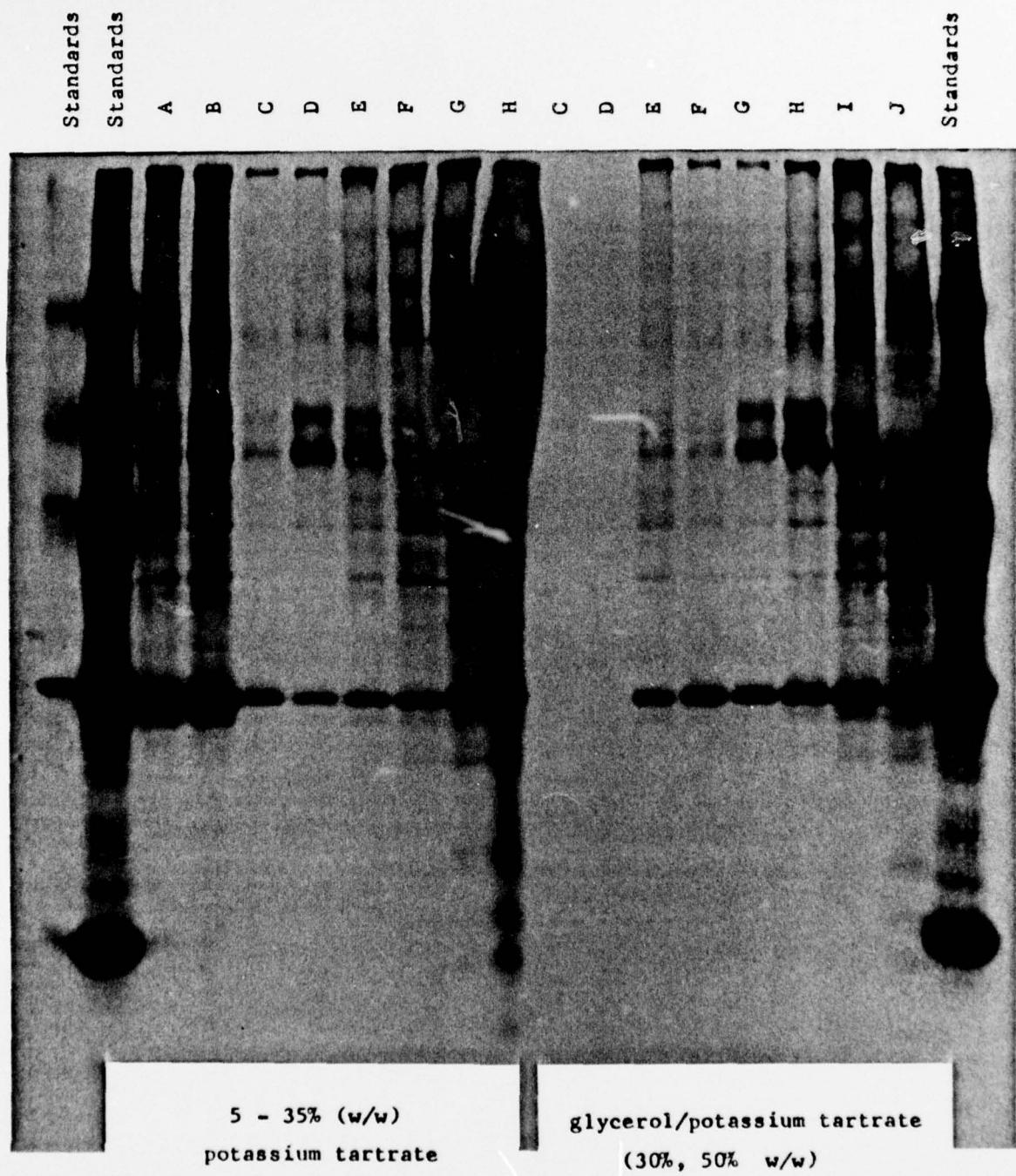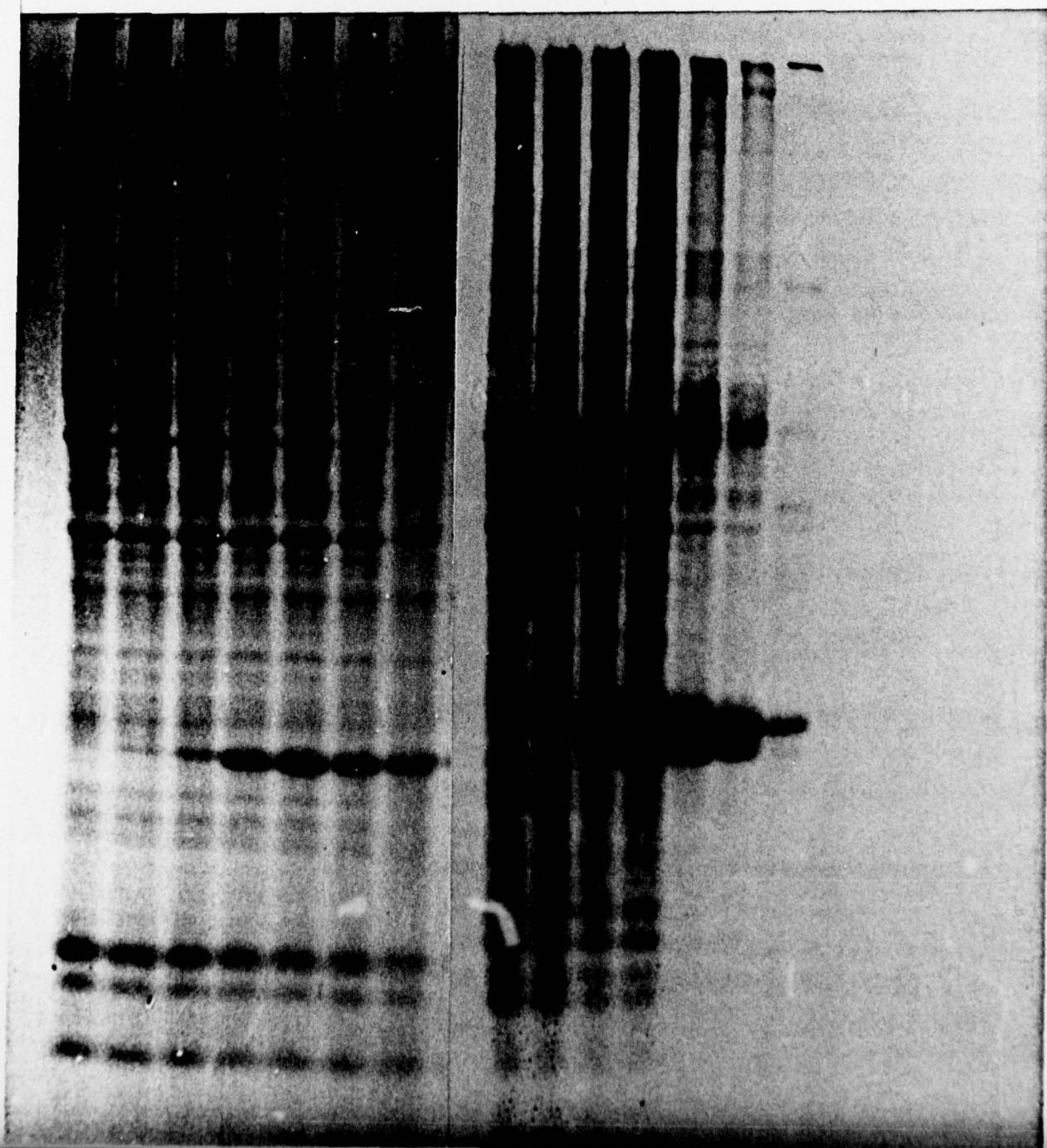
Figure 17

IMMUNOPRECIPITATION OF PUNTA TORA VIRUS PROTEINS   Figure 18

Figure 19

Standards

Inf. lysate #1

Inf. lysate #2

Inf. lysate #3

Inf. lysate #4

Inf. lysate #5

Control lysate #1

Control lysate #2

Control lysate #3

Control lysate #4

Control lysate #5

Inf. media pellet #4

Control media pellet #3

Standards

Mol. Wt. (X10^3)

12

69

46

30

12
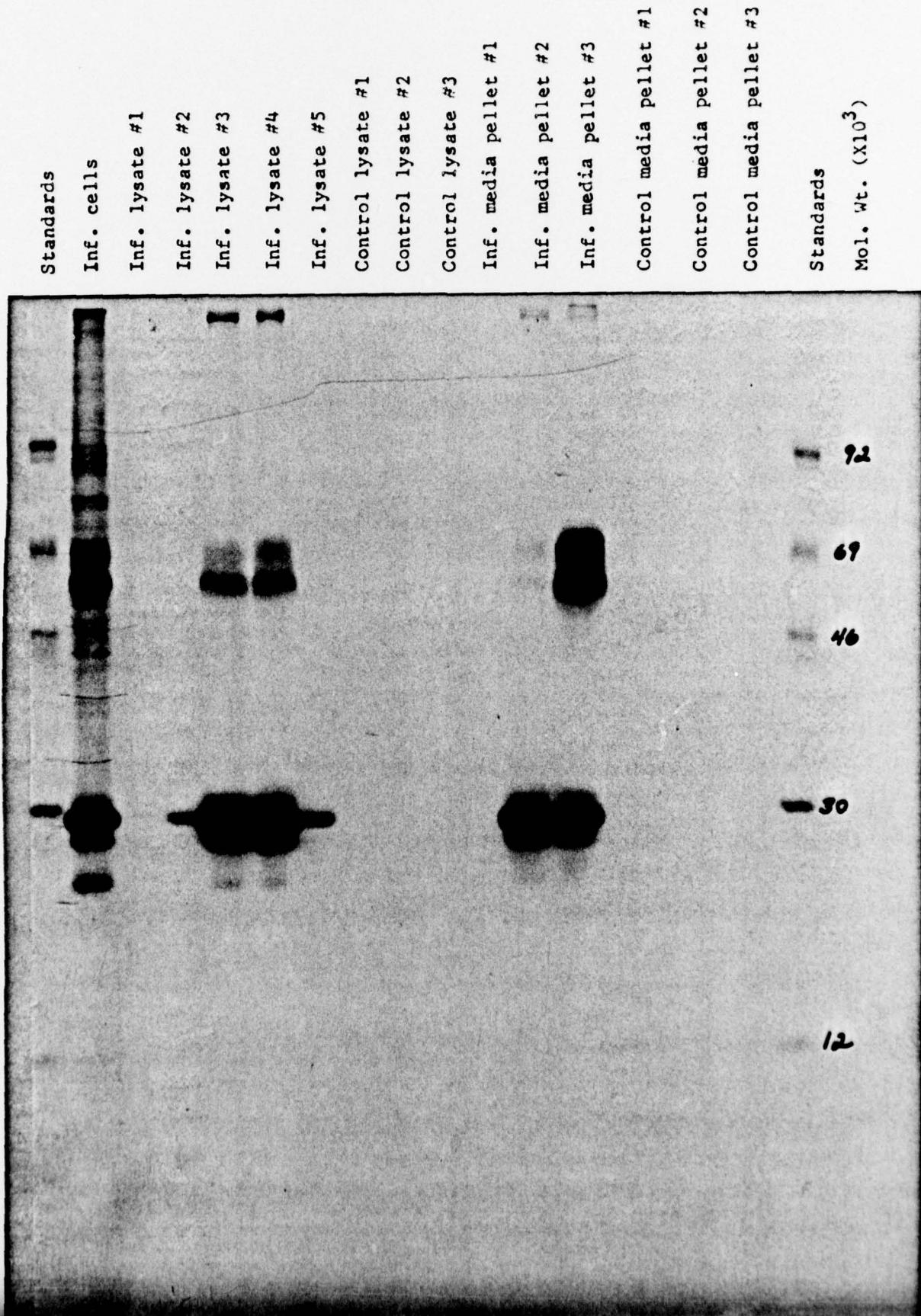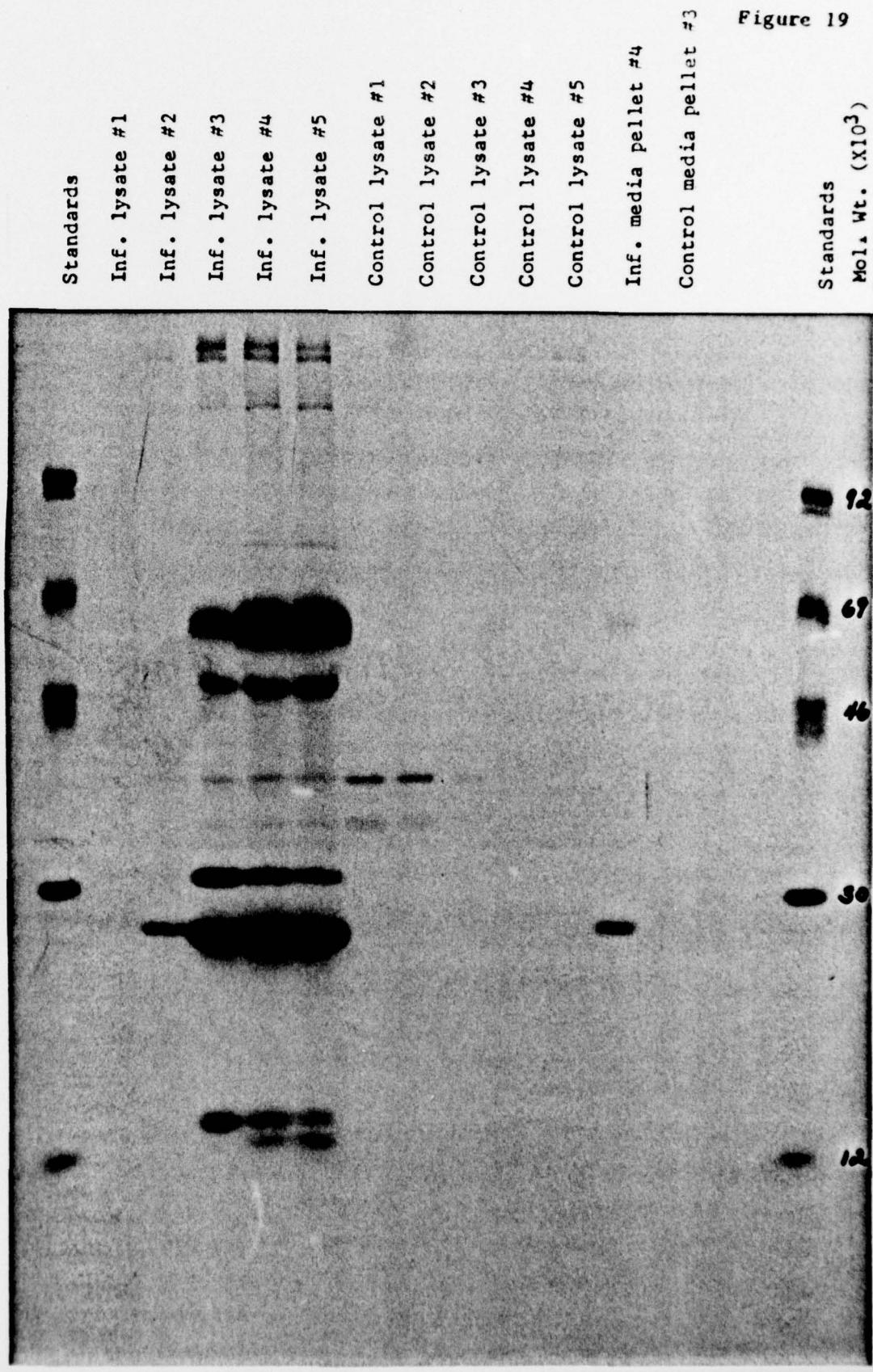
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